

THE ETIOLOGY AND PATHOGENESIS OF GREEN TURTLE
FIBROPAPILLOMATOSIS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1995

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by

Lawrence Henry Herbst

To my wife, Maria, and my two children, Thomas and Lisa, who have supported me as I have pursued my dreams and who have sacrificed the most to allow me that luxury. To my parents who taught me the importance of family and the value in doing what you love.

ACKNOWLEDGMENTS

I want to express my deepest appreciation to Dr. Paul A. Klein, chairman of my graduate committee, for the advice, assistance, encouragement, support, patience, and friendship that he has shown me throughout this project. I am also very grateful to Dr. Elliott R. Jacobson, cochairman of my graduate committee, for giving me the opportunity and encouraging me to conduct this project. Special thanks also go to Dr. Alan B. Bolten, Dr. Ammon B. Peck, Dr. Sheldon M. Schuster, and Dr. John P. Sundberg, members of my graduate committee, for their advice, assistance, and support.

A substantial part of this project would not have been possible without the facilities and dedicated support provided by Richie Moretti and Tina Brown of The Turtle Hospital, Marathon, FL. Their sincere concern for the environment and for endangered species has been amply demonstrated by their commitment of time, labor, and money to provide for the housing, husbandry, and rehabilitation of marine turtles. I also thank veterinarians, Dr. Lisa Bramson and Dr. Beatrice Lopez, for providing clinical care of research turtles in my absence.

Many others have contributed in various ways to the

success of this research project. I especially thank George Balazs (National Marine Fisheries Service, Southwest Fisheries Laboratory, Honolulu, HI) for his continued enthusiasm and interest in the fibropapilloma problem in green turtles. Through his efforts, significant funding for these studies were made available. I also thank Earl Possardt, Ren Lohoefer, and Sandra McPherson of the U.S. Fish & Wildlife Service for their support of this project. The Florida Department of Environmental Protection (DEP) provided permits to conduct these studies. In addition, the cooperation and assistance of DEP personnel, especially Barbara Schroeder, Allen Foley, Carrie Crady, and Blair Witherington, in providing information about and access to free-ranging or stranded turtles was extremely helpful. Special thanks go to Dr. Llewellyn Ehrhart, University of Central Florida, Orlando, FL, and his students and field assistants, especially Dean Bagley and Steven Johnson, for collecting blood, biopsy samples, and eggs. Eric Martin and Barbara Schroeder also provided turtle eggs. Jim and Fern Wood of Cayman Turtle Farm, Ltd, Grand Cayman, British West Indies, made captive turtles available for antibody studies and carried out immunizations. USAir Corporation transported hatchlings from Gainesville to Marathon free of charge. Sea World, Orlando, FL, provided access to turtles in their care for blood sampling. Dr. Karen Bjorndal and Dr. Alan Bolten of the Archie Carr Center for Sea Turtle Research provided

biological samples and allowed me to incubate eggs and house hatchlings in their laboratory. Alan Bolten also provided valuable assistance in negotiating the paperwork associated with permits and work orders. I thank the personnel of the Hybridoma Core Laboratory and Immunological Analysis Core, Interdisciplinary Center for Biotechnology Research (ICBR), especially Diane Duke, Linda Green, Cathy McKenna, and Isabella Schumacher for their assistance. I also thank Dr. Ratna Chakrabarty (Gene Expression Core, ICBR) for performing the preliminary differential message display analysis. Dr. Brian Gray (Cytogenetics Laboratory) karyotyped cell lines. Dr. Ellis Greiner (Department of Pathobiology) sorted and identified trematode parasites from green turtles. Dr. Bruce Homer (Department of Pathobiology) allowed me to work at times in his laboratory. Special thanks are due to Betty Hall for teaching me immunohistochemistry techniques. Dr. Leonard Shultz and Dr. John P. Sundberg (The Jackson Laboratory, Bar Harbor, ME) provided immunodeficient mice for tumorigenicity studies.

This project was supported by grants from SAVE-A-TURTLE, Islamorada, FL, a joint contract from the U.S. Fish & Wildlife Service, Department of the Interior and the National Marine Fisheries Service, Southwest Fisheries Science Center, NOAA, Department of Commerce (RWO No. 96), and a training fellowship from the National Institutes of Health (National Center for Research Resources RR07001).

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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By

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December, 1995

Chairperson: Paul A. Klein
Major Department: Department of Pathobiology

Green turtle fibropapillomatosis (GTFP) is a threat to populations of Chelonia mydas worldwide. This project attempted to characterize the etiology and to describe the pathogenesis of GTFP. Transmission studies showed that tumors could be induced in recipient turtles by inoculation with twice frozen and thawed cell-free homogenates prepared from spontaneous tumors. Tumors were not induced by inoculation with intact spirorchid ova nor were spirorchid ova found in any experimentally induced tumors. Oncogenicity of tumor homogenates passed through 0.45 μm but not 0.2 μm filters, and was destroyed by chloroform. Some spontaneous and experimentally induced tumors had epidermal eosinophilic

intranuclear inclusions, which contained herpesvirus-like particles. Attempts to culture this virus on 2 reptilian cell lines were unsuccessful. Particles resembling herpesvirus were found in pooled isopycnic gradient fractions of one transmission-positive tumor preparation, but were not tumorigenic. Green turtle antibody class-specific monoclonal antibodies, developed for the detection of turtle antibody responses to putative GTFP agents, were used with a proven herpesvirus-specific turtle antiserum, to demonstrate herpesvirus antigens in spontaneous and induced tumors. Tissue sections containing herpesvirus were also used to screen plasma samples for antibody reactivity to herpesvirus antigens by immunohistochemistry. Antibody reactivities to herpesvirus developed in all experimental transmission-positive turtles, but not in controls or transmission-negatives. A strong association between antibody reactivity to herpesvirus and clinical GTFP was also found in free-ranging turtles. In contrast, antibody reactivity to spirorchid trematodes was not associated with clinical GTFP. The transformed phenotype of GTFP-derived fibroblast cultures was demonstrated using tumorigenicity assays and preliminary studies showed differences in mRNA expression between matched pairs of normal skin- and GTFP-derived cell lines. Although the pathogenesis of GTFP can be explained by herpesvirus, proof that herpesvirus causes GTFP will require reproduction of the disease in turtles with purified virus, or

demonstration of herpesviral gene sequences among these differentially expressed messages in GTFP cell lines and in transmission positive tumor homogenates, that can transform normal fibroblasts to the tumorigenic phenotype.

CHAPTER 1 INTRODUCTION

Reports of neoplasia in chelonians are relatively uncommon (Billups & Harshbarger, 1976; Jacobson, 1980; 1981a; Machotka, 1984). For example, 24 neoplastic conditions in 19 turtle species, mostly represented by single case reports, were compiled by Machotka (1984). Cutaneous papillomas, fibromas, and fibropapillomas in green turtles, Chelonia mydas, however, were reported commonly (Machotka, 1984). These three proliferative lesions in Chelonia mydas are the hallmarks of Green Turtle Fibropapillomatosis (GTFP) and the number of observed cases has continued to increase while only 2 new case reports of neoplasia in other turtle species have subsequently been published (Frye et al., 1988; Machotka et al., 1992). Recent documented increases in GTFP prevalence and the spread of GTFP to locations where it had not been observed previously make GTFP the most common neoplastic disease of reptiles and a significant threat to endangered green turtle populations. Consequently, research to determine the cause of GTFP and find ways to reduce the impact of this disease has been listed as a priority in recovery plans for green turtles (Balazs et al., 1990; National Marine Fisheries Service & U.S. Fish and Wildlife Service, 1991).

More recently, fibropapilloma-like lesions have been reported in other marine turtle species, including loggerhead turtles, Caretta caretta (Llewellyn Ehrhart, University of Central Florida, Orlando, FL 32816, pers. comm.; Barbara Schroeder, Florida Marine Research Institute, Tequesta, FL 33469, pers. comm.), olive ridley turtles, Lepidochelys olivacea (Any Chaves, Universidad de Costa Rica, San Jose, Costa Rica, pers. comm.; Pamela Plotkin, Texas A&M University, College Station, TX 77843, pers. comm.) and flatback turtles, Natator depressus (Limpus & Miller, 1994), raising concerns about the potential impact of these diseases on all marine turtle populations.

The purpose of this research project has been to identify the cause of GTFP, to lay the groundwork for understanding the pathogenesis of this disease, and to begin to develop practical diagnostic tests for use in management applications and in ecological studies (epizootiology) of GTFP. This research has addressed the important questions that will lead to understanding of the impact of this disease on worldwide green turtle populations.

The specific objectives were as follows:

(1) To review current knowledge about GTFP and evaluate the various proposed hypotheses about its etiology (Chapter 2).

(2) To begin to develop the immunological tools needed to study the immune response to fibropapilloma cells or to

putative etiologic agents. The initial focus was to produce monoclonal antibodies specific for the known classes of green turtle immunoglobulins (Chapter 3).

(3) To determine whether GTFP can be transmitted experimentally and is therefore caused by an infectious (transmissible) agent (Chapter 4).

(4) To identify and characterize the etiologic agent (Chapter 5).

(5) To rule out alternative infectious etiologies (Chapters 4 & 5).

(6) To begin to describe the pathogenesis of GTFP based on histopathology, experimental findings, and identified serologic and epizootiologic associations (Chapters 6 & 7).

(7) To develop the techniques to distinguish cultured GTFP-derived, i.e., transformed fibroblasts from normal fibroblasts, as a basis for in vitro studies on the molecular mechanisms of fibroblast proliferation, the most salient feature of GTFP. In addition, to search for GTFP cell-specific antigens or genes in these cell lines for use in diagnostic test development (Chapter 8).

CHAPTER 2
REVIEW OF LITERATURE ON GREEN TURTLE FIBROPAPILLOMATOSIS

Historical Perspective

Cutaneous papillomas, fibromas, and fibropapillomas were first described by Smith and Coates (1938) in a captive green turtle, Chelonia mydas, at the New York Aquarium that had been captured near Key West, Florida, two years previously. Two other green turtles and 2 loggerheads that were housed with this animal did not have lesions. Smith and Coates (1938) also found fibropapillomas in 3 of 200 free-ranging green turtles (27-91 kg) that were captured off of Key West. That same year, Lucké described similar tumors from a green turtle caught off Cape Sable, Florida (Lucké, 1938). Masses were located on the tail, flippers, axillae, neck, eyelids, and corneas. Schlumberger and Lucké (1948) subsequently described fibropapillomas from 3 Florida green turtles and found numerous fibrous masses within the lungs of one turtle. In 1958, Hendrickson noted the occasional occurrence of fibrous masses on nesting females in Sarawak and Malaya (Hendrickson, 1958). The first confirmed case of GTFP in Hawaii occurred in 1958 and was a juvenile green turtle captured by local fisherman in Kaneohe Bay, Oahu (Balazs, 1991). A survey of local fishermen conducted by Balazs (1991)

suggests that GTFP was rare to nonexistent prior to this. Since this first report, green turtles with fibropapillomas have been reported with increasing frequency from Hawaii (Balazs, 1991; George Balazs, National Marine Fisheries Service, Southwest Fisheries Center, Honolulu, Hawaii 96822, pers. comm.). In 1980 an outbreak of fibropapillomatosis occurred in a breeding group of adult green turtles at Cayman Turtle Farm, Ltd, Grand Cayman, British West Indies (Jacobson, 1981b; Jacobson et al., 1989). The outbreak began in wild caught adults but subsequently developed over several years in farm raised turtles as well. Ehrhart (1991) documented the first cases of GTFP in the Indian River Lagoon, Florida, in 1982. Netting surveys within the northern portion of the Indian River Lagoon system (Mosquito Lagoon) had been conducted since 1977 without encountering any green turtles with fibropapillomas. However, when the study area was shifted to the central portion of the system (Indian River) in 1982, affected turtles were encountered immediately. A review of late 19th century accounts of the Florida east coast green turtle fishery and of reports on Indian River Lagoon turtles published between 1978 and 1983 failed to yield any record of GTFP prior to this (Ehrhart, 1991; Ehrhart et al., 1986). Continued monitoring at this site since 1982 has revealed GTFP prevalences around 50%.

Description of GTFP

Gross Pathology

Green turtle fibropapillomatosis (GTFP) is characterized by single to multiple raised cutaneous masses ranging from 0.1 cm to greater than 30 cm in diameter. Individual masses may be either verrucous or smooth and either sessile or pedunculated. Large masses are often ulcerated. Cutaneous fibropapillomas are usually found on the soft skin but may be found anywhere on the turtle's body, including carapace and plastron. Common sites for GTFP are the flippers, neck, chin, inguinal and axillary regions, and tail base (Figure 2-1). Ocular GTFP is common, with masses arising from the bulbar conjunctiva, limbus, cornea, or mucocutaneous junction of the eyelids (Brooks et al., 1994; Jacobson et al., 1989; Lucké, 1938; Smith & Coates, 1938) (Figure 2-2). Tumor pigmentation is usually related to the pigmentation of the skin at the site of origin.

Visceral tumors (Figure 2-3) have been found at necropsy in some green turtles with cutaneous fibropapillomatosis (Jacobson et al., 1991; Norton et al., 1990; Schlumberger & Lucké, 1948; Williams et al., 1994). Schlumberger and Lucké (1948) discovered numerous spherical 3-5 cm masses in the lungs of one green turtle. Norton et al. (1990) observed multiple firm white nodules in both kidneys from a juvenile green turtle with extensive cutaneous fibropapillomatosis collected in the Florida Keys. Jacobson et al. (1991)



Figure 2-1. Cutaneous fibropapillomatosis in the green turtle, *Chelonia mydas*. This juvenile stranded in December 1993 near Key West, Florida in severely debilitated condition as evidenced by the sunken plastron. Multiple tumors were found on the neck, front and rear flippers, axillary and inguinal areas, perineum, and covering both eyes.



Figure 2-2. Ocular fibropapillomatosis in green turtles, Chelonia mydas. (Top) Left eye with multiple fibropapillomas originating from bulbar and palpebral conjunctiva, limbus, and cornea. (Bottom) Right eye of a second turtle with large fibromas arising from the palpebral and bulbar conjunctiva.



Figure 2-3. Examples of visceral tumors found in some green turtles with severe cutaneous fibropapillomatosis. (Top) Kidneys with multiple, irregularly shaped, firm, white nodules (arrows) ranging from 0.5 to 3 cm in diameter bulging from the surface. (Bottom) Lungs with multiple nodules ranging from 0.2 to 5 cm in diameter. Lung nodules are well demarcated, smooth, and either firm and white (fibromatous), or gelatinous and translucent (myxomatous). Myxomatous nodules (arrow) appear to arise from fibromatous nodules.

examined 2 turtles with GTFP and, in one animal, found several discrete firm, white foci up to 1 mm diameter on the surface of one kidney and multiple discrete 1-4 cm diameter nodules in the other. They also found similar nodules 1-2 cm diameter within both lungs. Williams et al. (1994) found lung and kidney nodules in 41% (7 of 17) of the green turtles examined from Puerto Rico. Approximately 17% (9 of 52) of the green turtles with severe cutaneous fibropapillomatosis presented for necropsy at a rehabilitation center have been found to have similar nodules in the lungs, kidneys, and other viscera (Herbst, pers. obs.; Richie Moretti & Tina Brown, The Turtle Hospital, Marathon, FL 33050, pers. comm.).

Histopathology

Several histologic descriptions of cutaneous GTFP have been published (Aguirre et al., 1994b; Brooks et al., 1994; Harshbarger, 1991; Jacobson et al., 1989; Lucké, 1938; Norton et al., 1990; Smith & Coates, 1938, 1939; Slumberger & Lucké, 1948; Williams et al., 1994). Cutaneous GTFP is described as papillary epidermal hyperplasia supported on broad fibrovascular stromal stalks (Figure 2-4). The ratio of epidermal to dermal proliferation varies among lesions. Masses in which both tissues are hyperplastic are termed fibropapillomas (Figure 2-4A) while others, comprised of proliferating dermal components with relatively normal epidermis, are termed fibromas (Figure 2-4B). Several authors have postulated that there is a developmental progression

from papilloma (early lesions) through fibropapilloma, to fibroma (chronic lesions) (Harshbarger, 1991; Jacobson et al., 1989; Lucké, 1938).

Varying degrees of orthokeratotic hyperkeratosis and acanthosis were consistent features in all studies (Aguirre et al., 1994b; Brooks et al., 1994; Harshbarger, 1991; Jacobson et al., 1989; Lucké, 1938; Norton et al., 1990; Smith & Coates, 1938, 1939; Schlumberger & Lucké, 1948). The degree of epidermal hyperplasia in GTFP varied from mild to moderate (7-15 cells thick) on skin tumors to extensive (up to 30 cells thick) on some conjunctival and palpebral masses (Brooks et al., 1994; Jacobson et al., 1989). Fibropapillomas with extensive epithelial hyperplasia often exhibit anastomosing rete ridges extending deep into the dermis. Epithelial cells in hyperplastic areas tend to be hypertrophied (Brooks et al., 1994; Jacobson et al., 1989). The fibrovascular stroma contains numerous well-differentiated fibroblasts arranged in a ground substance containing compact bundles of collagen fibers. Fibroblasts and collagen bundles tend to be haphazardly arranged, are more numerous than in normal dermis, and are more dense near the basement membrane (Brooks et al., 1994; Jacobson et al., 1989). Various amounts of collagen and mucopolysaccharide

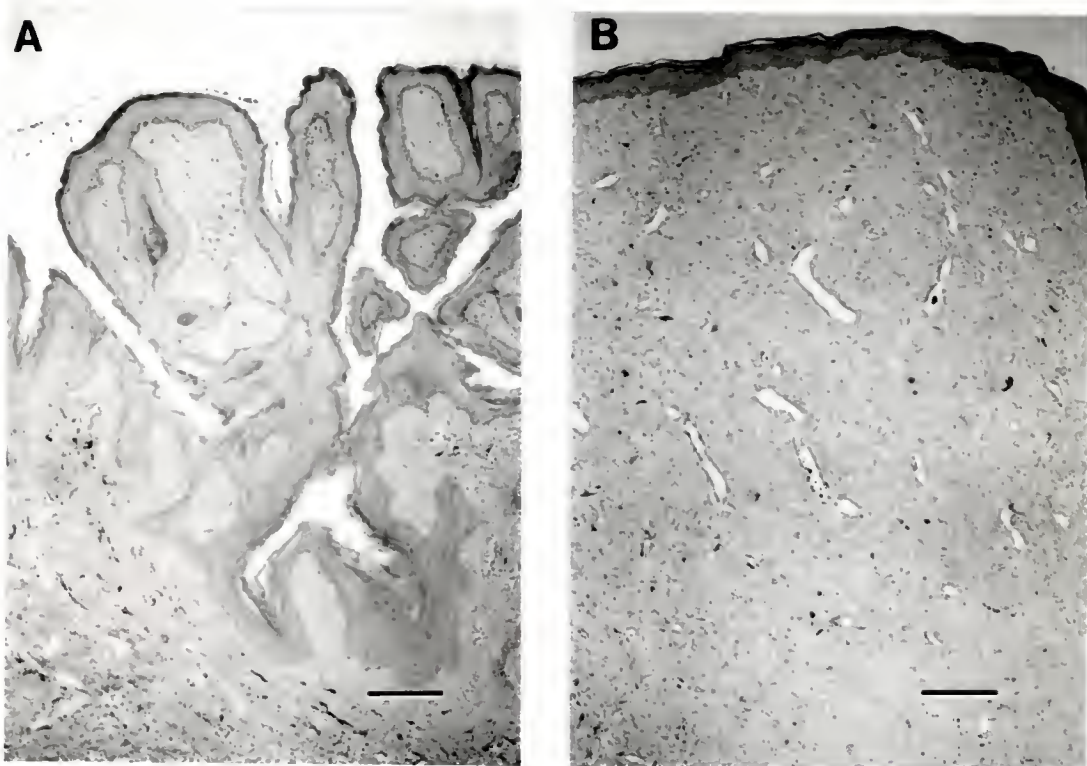


Figure 2-4. Variation in histologic appearance of cutaneous fibropapillomatosis. (A) Fibropapilloma showing typical arborizing pattern of papillary epidermal hyperplasia supported by fibrovascular stroma. (B) Fibroma showing extensive fibrovascular proliferation covered by relatively normal epidermis. (H&E, scale bars = 250 μ m).

ground substance have been demonstrated in cutaneous tumors by trichrome and alcian blue staining (Norton et al., 1990). Nerves and numerous small blood vessels are found within the stroma. Fibropapillomas examined in several studies show no malignant or anaplastic changes and few mitotic figures (Smith & Coates, 1938; Williams et al., 1994). The benign nature of GTFP has been confirmed by flow cytometry studies (Papadi et al., 1995).

Some histologic features identified in cutaneous tumors have not been reported consistently and may be incidental findings. Trematode (Spirorchidae) eggs surrounded by epithelioid macrophages and multinucleate giant cells were found within the dermal capillaries of some fibropapillomas (Aguirre et al., 1994b; Brooks et al., 1994; Harshbarger, 1991; Jacobson et al., 1989; Jacobson et al., 1991; Norton et al., 1990; Smith & Coates, 1939; Williams et al., 1994). In some lesions containing eggs, eosinophilic granulocyte infiltrates were also observed (Smith & Coates, 1939). Epithelial cells in the stratum spinosum and outer layers of epidermis were hypertrophic and vacuolated in some GTFP specimens. In these areas, amphophilic intranuclear inclusions were sometimes observed (Aguirre et al., 1994b; Jacobson et al., 1989). Jacobson et al. (1991) found eosinophilic intranuclear inclusions containing herpesvirus-like particles within some superficial epidermal cells undergoing intracytoplasmic vacuolation and ballooning

degeneration. Lymphocytic perivascular infiltrates have been described in several studies (Jacobson et al., 1989; Smith & Coates, 1938). Cleft formation at the dermal-epidermal junction has also been noted in fibropapillomas examined by Jacobson et al. (1989).

Visceral tumors were composed of proliferating fibrous tissue compatible with the dermal component of cutaneous GTFP (Jacobson et al., 1991; Norton et al., 1990; Schlumberger & Lucké, 1948). Lung nodules described by Schlumberger and Lucké (1948) were covered by ciliated columnar epithelium. The renal nodules described by Norton et al. (1990) were sharply demarcated from surrounding renal tissue and covered by renal capsule at the surface. Normal renal tubules were found scattered throughout the proliferating connective tissue.

Epizootiology of GTFP

Epizootiology is the study of the temporal and spatial patterns of disease expression in animal populations and includes efforts to identify etiology, describe incidence and prevalence, morbidity and mortality, routes of natural exposure or transmission, and the conditions that lead to disease outbreaks (epizootics). This information is needed to understand the full demographic impact of GTFP on wild turtle populations.

Epizootiologic studies of GTFP have been hampered by several factors. First, there are no diagnostic tests to

detect exposure and early (preclinical) disease because an etiologic agent has not been identified. Thus, all prevalence data are based on observation of gross cutaneous tumors. Second, because green turtles are migratory and long lived, taking between 20 and 50 years to reach sexual maturity (Balazs, 1982; Frazer & Ehrhart, 1985; National Marine Fisheries & US Fish and Wildlife, 1991), it is difficult to sample specific life history stages such as the post hatching pelagic phase and impossible to conduct longitudinal studies of cohorts. Third, attempts to correlate disease prevalence with assorted biotic and abiotic factors are hampered by the geographic scale over which field surveys need to be conducted and by limited human and fiscal resources. Consequently, surveillance for GTFP and monitoring of potentially relevant biotic and abiotic factors has been limited.

Even in well-monitored sites, sampling methods introduce biases that affect prevalence estimates. Most field studies are conducted on feeding grounds or nesting beaches, and it is therefore not surprising that post pelagic juveniles and adult females are over represented in the prevalence reports. Nesting beach surveys underestimate the true prevalence of GTFP in adult females because debilitated turtles are less likely to nest and are therefore not sampled (Limpus & Miller, 1994). Field surveys and fisheries that employ tangle nets tend to selectively sample larger turtles because small

turtles are not caught in the mesh. Surveys based on stranded sea turtles may overestimate the prevalence of severe debilitating disease. Cold stunning events, and sampling methods that use direct, in-water captures, provide the least biased population samples. The reader is asked to keep these caveats in mind when evaluating the information presented below.

Geographic Distribution

Presently, GTFP has a global circumtropical distribution (Figure 2-5 and Table 2-1). GTFP has been reported from all major oceans including the Atlantic (Florida, Bahamas, Brazil), Caribbean (Cayman Islands, Puerto Rico, Virgin Islands, Barbados, Venezuela, Colombia, Nicaragua, Costa Rica, Panama, Belize), and Indo-Pacific (California, Hawaii, Australia, Sri-Lanka, Seychelles, Sarawak, Malaya, Japan) (Balazs, 1991; Balazs & Pooley, 1991; Gamache & Horrocks, 1991; Hendrickson, 1958; Jacobson, 1990; Jacobson et al., 1989; MacDonald & Dutton, 1990; Limpus & Miller, 1994; Williams et al., 1994; Karen Bjorndal & Alan Bolten, University of Florida, Gainesville, FL 32611, pers. comm.; Cynthia Lageaux, University of Florida, Gainesville, FL 32611, pers. comm.; Anne Meylan, Florida Marine Research Institute, St. Petersburg, FL 33701, pers. comm.; Jean Mortimer, University of Florida, Gainesville, FL 32611, pers.

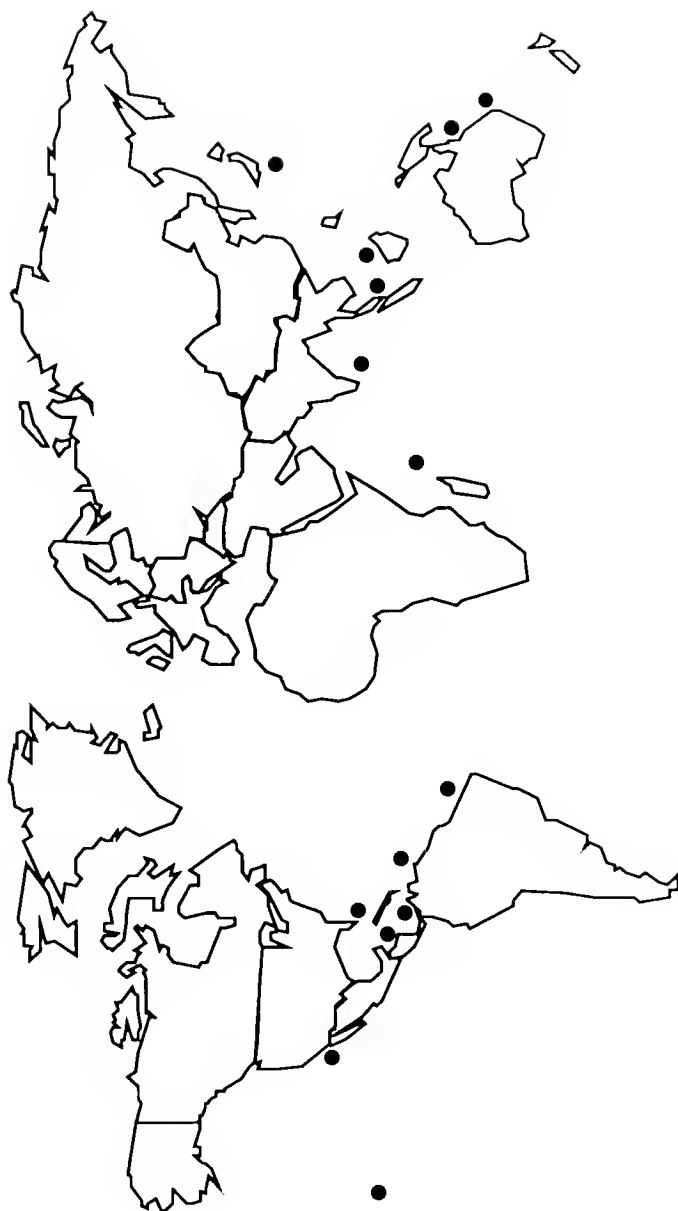


Figure 2-5. Circumtropical distribution of green turtle fibropapillomatosis (GTFP). Localities where GTFP has been reported are marked in black dots. Although monitoring is sporadic in many areas, GTFP has been reported from every major ocean basin in which green turtles, Chelonia mydas are found.

comm.). Unfortunately, there are insufficient data to reconstruct the temporal and spatial pattern of disease spread among regions. The early reports from Florida (Smith & Coates, 1938) and Malaysia (Hendrickson, 1958) suggest that the disease may have always had a worldwide, albeit sporadic distribution.

Prevalences

The prevalence of GTFP varies among locations and from year to year. Table 2-1 summarizes the available prevalence data from several field studies. The earliest published prevalence estimate (1.5 %) was from a survey conducted in 1938 of turtles captured in the Key West, Florida fishery (Smith & Coates, 1938). Most population surveys, however, have been conducted since 1975. In these surveys, little or no disease was found prior to 1982, but prevalences rose rapidly in the 1980s and have remained elevated. Part of this pattern may reflect an increased awareness of the disease, but may also reflect a real increase in the prevalence and severity of GTFP over time.

Prevalences in well monitored feeding ground sites range from 0% in Inagua, Bahamas (Bjorndal & Bolten, pers. comm.), Bermuda (Meylan, pers. comm.) and offshore reef sites in Australia (Limpus & Miller, 1994), to 92% in Kaneohe Bay, Hawaii (Balazs, 1991). Prevalences may vary greatly between demographically matched populations over very short distances (< 1 km), as seen when comparing the prevalence of GTFP in

Table 2-1. Prevalences of GTFP among different populations of free-ranging green turtles

Locality	Turtle Habitata	Impact of Human Activity ^b	Prevalence of GTFP (%)	Sample Period	Sample Method	Reference
Florida, Gulf Coast			50	1992 (1st record, 1985)	Stranding recovery	Teas (1991)
Florida, Florida Bay	Seagrass flats	Agricul- ture, urban	70	1990-93	Hand capture, net	Schroeder (pers. comm.)
Florida, Florida Keys	Reef / seagrass flats		1.5	1938	Fishery survey	Smith & Coates (1938)
Florida, Florida Keys			20-60	1980-90	Stranding recovery	Teas (1991)
Florida, Atlantic Coast			10	1980-90	Stranding recovery	"
Florida, Wabasso Beach	Ocean, inshore reef		0	1988-93	Net	Ehrhart (1991, pers. comm.)
Florida, Mosquito Lagoon	Lagoon, seagrass flats	Relatively protected natural area	0	1975-81	Net	Ehrhart et al. (1986), Ehrhart (1991)
			0	1977, 1981	Cold stun recovery	
			29	1985	Cold stun recovery	
			1.6	1990	Cold stun recovery	

Table 2-1--continued.

Locality	Turtle Habitat ^a	Impact of Human Activity ^b	Prevalence of GTFP (%)	Sample Period	Sample Method	Reference
Florida, Indian River	Lagoon, seagrass flats	Agriculture, urban, industry	20-61	1982-1993	Net	Ehrhart (1991, pers. comm.)
Bermuda	Reef / seagrass flats	Minimal (pristine)	0	1968-1993	Net	Meylan (pers. comm.)
Bahamas, Inagua	Tidal bay, seagrass flats	Minimal (pristine)	0	1974-1993	Hand capture	Bjorndal & Bolten (pers. comm.)
Nicaragua, Porta Cabras	Reef / seagrass flats	Minimal	< 5	1993	Fishery survey	Lageaux (pers. comm.)
Panama, Bocas del Toro, Chiriqui Lagoon	Lagoon, seagrass flats	Minimal	35	1989-93	Net	Meylan (pers. comm.)
Puerto Rico			17	1988-92 (1st record, 1987)	Stranding recovery	Teas (1991)
Barbados, Barclay's Park	Nearshore feeding ground	Agriculture	90	1990 (1st record, 1982-83)	Net, fishery	Gamache & Horrocks (1991)
Hawaiian Islands			26-60	1983-93	Stranding recovery	Balazs (1991, pers. comm.)
Hawaii, Kiholo Bay	Bay, reef / algae flats	Minimal	0	1987-90	Hand capture	Balazs (1991)

Table 2-1--continued.

Locality	Turtle Habitata	Impact of Human Activityb	Prevalence of GTFP (%)	Sample Period	Sample Method	Reference
Hawaii, Punaluu Bay	Bay, reef / algae flats	Minimal	1	1976-93 (1st record, 1984)	Hand capture	Balazs (1991, pers comm)
Molokai, Palaa	Reef / algae flats	Agricul- ture (minimal)	0 1-53	1982-85 (1st record, 1985)	Net	Balazs (1991, pers. comm.)
Oahu, Kaneohe Bay	Bay, reef	Urban	49-92	1987-93 1989-1991 (1st record, 1958)	Hand capture	Balazs (1991)
Oahu, Waikiki Beach	Reef	Urban	9	1990-93	Hand capture	Balazs et al. (1994)
French Frigate Shoals	Nesting beach		7-12	1988-92	Beach survey	Balazs (1991, pers. comm.)
Pearl / Hermes Reef	Reef	Minimal (pristine)	0	1982-87	Hand capture	Balazs (1991)
Midway Island	Reef	Urban (military infra- structure)	0	1969-78 (1st record, 1990)	Hand capture	Balazs (1991)
Australia, Torres Strait	Reef	Pristine	0	1977-80	Fishery survey	Glazebrook & Campbell (1990b)
Australia, Heron Island & Wistari Reefs	Outer- barrier reef	Pristine	0	1968-92	Hand capture	Limpus & Miller (1994)

Table 2-1--continued.

Locality	Turtle Habitat ^a	Impact of Human Activity ^b	Prevalence of GTFP (%)	Sample Period	Sample Method	Reference
Australia, Clack Island Reef	Inner-shelf reef	Pristine	0	1988-90	Hand capture	Limpus & Miller (1994)
Australia, Hazelwood Island Reef	Inner-shelf reef	Pristine	0	1989	Hand capture	Limpus & Miller (1994)
Australia, Green Island Reef	Inner-shelf reef	Pristine	0	1988-90	Hand capture	Limpus & Miller (1994)
Australia, Shoal Water Bay	Inshore seagrass flats	Relatively protected natural area	2-3	1988-90	Hand capture	Limpus & Miller (1994)
Australia, Repulse Bay	Inshore seagrass flats	Agriculture	0-22	1988-90 (1st record, 1989)	Hand capture	Limpus & Miller (1994)
Australia, Moreton Bay	Inshore seagrass flats	Urban, industry	8	1990	Hand capture	Limpus & Miller (1994)
Seychelles	Nesting beach		0	1981-92	Beach survey	Mortimer (pers. comm.)
Aldabra Island	Feeding ground	Minimal (pristine)	0	1981-92	Hand capture	Mortimer (pers. comm.)

^a Habitat descriptions are those provided by each reference source.^b Qualitative descriptions of impacts of human activity are those provided by each reference source.

the Indian River (approximately 50%) with that from the adjacent near-shore Sabellariid worm reef at Wabasso Beach (0%) (Ehrhart, 1991; pers. comm.).

Seasonality

There is a seasonal pattern in the prevalence of GTFP among stranded green turtles in Florida with more affected turtles stranding in the winter months (Wendy Teas, Southeast Fisheries Science Center, NMFS, Miami, FL, 33149, pers. comm.). Anecdotal reports indicate that tumors grow rapidly in summer and are quiescent in the winter in response to water temperature (Moretti & Brown, pers. comm.). Thus, tumors may grow rapidly in summer and may reach a size that is debilitating by autumn. The onset of colder water temperatures in winter may further stress GTFP affected turtles sufficiently to cause the winter stranding peak.

Demographic Patterns

GTFP appears to effect certain age and size classes of turtles more than others. GTFP is rare (0-12%) among nesting adult females and lesions tend to be focal and mild (Balazs, pers. comm.; Ehrhart, pers. comm.), although these data are probably biased (see above). In Hawaiian feeding ground sites, intermediate sized turtles, measuring 40-90 cm straight carapace length (SCL), were more frequently and more severely affected than other size classes (Balazs, 1991). Ehrhart (1991) and Schroeder (pers. comm.) found similar results in Indian River and Florida Bay, respectively.

Turtles weighing between 10 and 30 kg were more likely to have GTFP and they were more severely affected than larger or smaller size classes (Ehrhart, 1991). Similarly, among stranded turtles in Florida, 93-98% are between 30 and 69.9 cm SCL (Teas, pers. comm.).

Juvenile green turtles enter near-shore feeding grounds after 2-5 years of pelagic existence. Too few pelagic stage juvenile green turtles have been examined to provide any information about GTFP prevalence in this life history stage. The consensus, however, is that juveniles develop GTFP after they have migrated into near-shore waters. This hypothesis is supported by the very low prevalence estimates for off-shore sites (Table 2-1) and the fact that GTFP has never been observed in any recent recruits from the pelagic environment to near-shore habitats (Balazs, 1986; Ehrhart, 1991; Limpus & Miller, 1994). Newly arrived juveniles are recognized by their small size (25-30 cm SCL, weight < 5 kg) and the lack of epibiota (algae, bryozoans, leeches, etc) on their carapaces as seen in older resident turtles (Ehrhart, 1991; Bolten & Bjorndal, 1992; Limpus & Miller, 1994). Although these small turtles are under-represented in net surveys, relatively unbiased samples of the near-shore juvenile turtle population have been obtained during cold stunning events. During one such event in 1985 in the Mosquito Lagoon, Florida, 145 cold stunned turtles were collected (Witherington & Ehrhart, 1985). Twenty-eight percent of these

turtles were recent recruits (< 5 kg) and none had GTFP even though GTFP prevalence in the overall sample was 29%.

There are several explanations for the absence of clinical GTFP among turtles that have recently immigrated to near-shore feeding grounds from the pelagic environment. One is that affected pelagic juveniles do not survive long enough to migrate to feeding grounds. Survivorship of healthy turtles through the 2-5 years of pelagic existence is already so low that it is unlikely that diseased turtles would survive. A second explanation is that the disease has a long latent period, so that clinical disease develops only in older juveniles after they have moved inshore, even though exposure to the etiologic agent(s) may occur in the pelagic zone. A third hypothesis is that the causative agent(s) or environmental conditions appropriate for disease expression are found only in some near-shore habitats, so that exposure occurs after juveniles are recruited to these sites.

The wide variation in GTFP prevalence among size/age matched populations of juvenile green turtles lends support to the last hypothesis. If the cause of GTFP were encountered in the pelagic zone and the pelagic juveniles assorted randomly among near shore sites, then one would expect the distribution of GTFP prevalences among these near-shore sites to be more uniform than is observed. Monitoring studies in the Indian River system provide the most convincing data because GTFP prevalence varies from 0% to approximately 50%

over a very short distance (< 1 km) between two demographically similar populations and there is documented movement of turtles from the low prevalence site (ocean) into the high prevalence site (lagoon) but not vice versa (Ehrhart, 1991; pers. comm.).

Habitat Associations

The field survey data summarized in Table 2-1 indicate that GTFP is more prevalent in shallow, near-shore ecosystems (lagoons, bays) and possibly most prevalent in areas that are impacted by human activities such as agricultural, industrial, and urban development within the catchment basin.

The strongest association of GTFP prevalence appears to be with habitat type (embayments). These marine environments may provide favorable physical conditions for either infectious or non-infectious disease agents. For example, certain sediment types may accumulate chemical contaminants and, combined with low flushing rates, could increase the level of exposure to chemical carcinogens or immunotoxins. These same sediment properties and hydrodynamic conditions may also favor the accumulation and maintenance of high concentrations of infectious agents. More variable water temperatures in shallow embayments could affect the rate of xenobiotic metabolism, tumor cell proliferation, immune system function, and pathogen replication. For example, thermal stress has been shown to exacerbate virus infection in hatchling green turtles (Haines & Kleese, 1977; Kleese,

1984) and modulate virus expression and tumor growth in Lucké renal adenocarcinoma (McKinnell, 1981, 1984; Zambernard & Vatter, 1966). Variable salinity in near-shore habitats may have similar stress effects.

Near-shore habitats may also provide an optimum biotic environment for survival and transmission of an infectious etiologic agent. Disease transmission could be enhanced by high population densities of vectors or intermediate host species. Feeding grounds may attract a high density of susceptible turtles which would facilitate the transmission of pathogens in a density dependent fashion, as has been shown for horizontally transmitted damselfish neurofibromatosis (Schmale, 1991) and the Lucké virus (McKinnell, 1981, 1984). Recruitment of susceptible turtles from many different breeding stocks into common foraging grounds may allow the exchange of many diseases, including GTFP, from exposed to naive individuals. Habitat differences in levels of other stressors such as concurrent infectious disease (parasites), and disturbance by human activity (fishing, boating, dredging) may render turtles more susceptible to or less able to recover from GTFP. These hypotheses provide the conceptual framework for future epizootiologic studies.

Clinical Course, Morbidity, and Mortality

Accurate estimates of the number of turtles that become clinically affected following exposure to the agent(s) that

cause GTFP are unavailable. The duration and course of clinical GTFP are poorly understood, primarily because individual turtles with fibropapillomas of known duration have not been available for longitudinal studies. A few green turtles have been held in captivity long enough to provide some generalizations about clinical course of the disease. Jacobson et al. (1989) held 6 immature turtles with multiple cutaneous GTFP in captivity for several months. Some tumors on some animals decreased in size while others increased in some animals when examined 4 months after capture. Ehrhart maintained 3 green turtles with GTFP in captivity for approximately 3 months (Ehrhart, 1991; Ehrhart et al., 1986). During that time one animal lost several tumors, a second developed 8 new tumors, and the third exhibited no changes in tumor burden. In these captive observation studies, the length of time that animals had the disease prior to capture was unknown.

Field mark and recapture studies also indicate a variable clinical course. For example, of 56 green turtles recaptured in the Indian River, 7% had tumors when first marked but had none at recapture, 14% contracted tumors between first capture and recapture, 38% had lesions both times, while 41% were free of lesions both times (Ehrhart, 1991). These data, while limited in number, support the conclusion that the clinical course is prolonged and that some individuals may spontaneously recover from disease.

Recapture rates are generally low, however, and there is no control over the time interval between capture and recapture. Studies of the temporal patterns of progression and regression of experimentally transmitted GTFP in captive turtles are needed.

Accurate estimates of the proportion of clinically affected turtles that die are unavailable. Cutaneous fibropapillomas can become large enough to interfere with locomotion and are easily entangled in discarded line. Ocular fibropapillomas (Figure 2-2) may occlude vision and those invading the cornea may cause secondary panophthalmitis with destruction of the globe (Brooks et al., 1994; Herbst, pers. obs.). Visceral fibromas (Figure 2-3) grow by expansion within the stroma of the affected organ and eventually disrupt normal organ functions. Cardiac dysfunction, buoyancy problems and respiratory compromise, hydronephrosis, gastrointestinal obstruction have all been observed or suspected causes of death in affected turtles (Balazs, pers. comm.; Herbst, pers. obs.; Moretti & Brown, pers. comm.). Many green turtles with multiple cutaneous fibropapillomas become severely debilitated (Figure 2-1). Blood chemistries and blood cell counts of severely affected green turtles confirm a general pattern of debilitation (Jacobson, 1987; Norton et al., 1990). Abnormalities include non-regenerative anemia, hypoproteinemia, electrolyte imbalances, uremia, and elevations in liver enzymes (Norton et al., 1990). The

cachexia may be caused by any combination of the following: inability to locate, ingest, or digest food, excessive energy demands for growth by proliferating tumors, increased energetic costs for locomotion, the physiological effects of certain cytokines such as tumor necrosis factor, mediated by the immune system, and/or concurrent disease such as spirorchidiasis. Whatever the mechanism(s), a number of animals become sufficiently debilitated by GTFP to strand (Balazs, 1991; Teas, 1991). In one rehabilitation center about 50% of green turtles that were still alive at stranding died despite extensive rehabilitation efforts (Moretti & Brown, pers. comm.).

Possible Causes of GTFP

The etiology of GTFP is unknown and its identification is one of the aims of this research project. Hypotheses about etiology can be proposed based on comparisons with similar tumors of known etiology from other species, the association of potential pathogens with GTFP, and from epizootiologic patterns. However, demonstration of causation requires rigorous experimentation to fulfill Koch's postulates. This is most easily accomplished with diseases caused by infectious agents. However, infectious disease expression may depend on a variety of host-related, pathogen-related, and environmental factors (Hanson, 1988).

GTFP has histologic features in common with benign cutaneous neoplasia, found in other vertebrates, such as

papillomas and fibromas (Pulley & Stannard, 1990), as well as hyperplastic conditions such as keloidosis (Caro & Bronstein, 1985) and exuberant granulation tissue (Smith et al., 1972). Thus, GTFP is consistent with either neoplasia or hyperplasia. The classification of GTFP as a neoplastic disease has been controversial (Harshbarger, 1984). In general, neoplasia may result from any of a variety of derangements at any point in the complex signalling and control network of normal cellular proliferation and differentiation (Bishop, 1991). The pathogenesis of neoplasia may involve multiple cumulative steps (Hunter, 1991; Peraino & Jones, 1989), with early steps causing unregulated proliferation and later events leading to malignancy. Similarly, hyperplasia can be caused by a variety of stimuli. Thus, identifying a single etiology and fulfilling Koch's postulates for GTFP may be difficult if not impossible.

Some potential causes of neoplastic and hyperplastic proliferative lesions in other vertebrate species include abiotic agents (ultraviolet light, chemical contaminants), and infectious biological agents (viruses, bacteria, metazoan parasites), with or without predisposing heritable genetic conditions. The following sections review and discuss evidence for or against the involvement of each of these factors in the etiology and pathogenesis of GTFP.

Environmental Factors

Ultraviolet light

Smith and Coates (1938) were the first to suggest a role for solar radiation in the pathogenesis of GTFP. Fifty years later there is mounting concern that ozone depletion is causing an increase in ultraviolet-B (290-320 nm) irradiation (Kerr & McElroy, 1993) and that this may be having pervasive effects in aquatic ecosystems (Hader, 1993) and on animal health (Van der Leun & De Gruijl, 1993). UV-B produces direct DNA damage by pyrimidine dimer formation (Anathaswamy & Pierceall, 1990). This may lead to mutation in cellular oncogenes and the development of neoplasia (Brash et al., 1991). UV-B also causes immunosuppression in experimental animals (Baadsgaard, 1991; Donawho & Kripke, 1991; Granstein, 1990; Noonan & DeFabo, 1992). The proposed mechanism involves pyridine dimer formation (Applegate et al., 1989; Kripke et al., 1992) and/or a trans to cis isomerization of urocanic acid in the skin following UV-B absorption (DeFabo & Noonan, 1983; Noonan & DeFabo, 1992). For example, trout exposed to levels of UV-B radiation within the ambient range recorded for mid-latitudes developed skin damage and became immunosuppressed, as evidenced by a high prevalence of fungal skin infections (Fabacher et al., 1994).

Increased UV-B exposure could occur in the shallow inshore waters where green turtles feed. However, GTFP prevalence varies too greatly over very short distances (as

in Ehrhart's study area) for UV-B to be the cause of GTFP. UV-B may be a cofactor in disease expression, however, and the role of UV-B in modulating the immune system of turtles deserves further investigation.

Chemical contaminants

A variety of chemical compounds have been shown to cause benign fibroepithelial proliferation and to have mutagenic and carcinogenic properties under experimental conditions (Anderson & Reynolds, 1989; Weisburger, 1989). The list of compounds is extensive but they seem to act by either of two basic mechanisms: (1) direct nucleic acid damage leading to genetic mutation (initiators), and (2) cellular damage or irritation leading to proliferation (promoters). As mentioned earlier, chemical effects may be one of many mechanisms involved in multistep carcinogenesis.

The involvement of chemical contaminants in naturally occurring neoplastic disease of lower vertebrates has been documented best in fish. The prevalence of liver pathology, including liver neoplasia in brown bullheads, Ictalurus nebulosus, was higher at contaminated sites than at relatively clean sites in several North American lakes and rivers (Baumann et al., 1987; Black, 1983; Bowser et al., 1990a). Disease prevalence was correlated with contaminant levels in fish in one study (Baumann et al., 1987) and with sediment levels in another (Black, 1983). Neoplastic lesions were induced experimentally by treating bullheads with

sediment extracts (Black, 1983). Similar associations between hepatic neoplasia, polluted sites, and sediment contaminant concentrations have been found for mummichogs, Fundulus heteroclitus in Chesapeake Bay (Vogelbein et al., 1990) and various bottom fish in Puget Sound (Malins et al., 1984). A similar association has been found between contaminated sites, in vitro mutagenesis of water and sediment extracts from those sites, and the prevalence of pigment cell neoplasia (Chromatophoromas) in croakers, Nibea mitsukurii (Kimura et al., 1984; Kinae et al., 1990). In addition, experimental application of chemical carcinogens reproduced the tumors in these fish. In most of these studies, polycyclic aromatic hydrocarbon (PAH) concentration was a major factor in the association between disease prevalence with contaminant levels. Similarly, PAHs have been implicated in the pathogenesis of cutaneous neoplasia in tiger salamanders, Ambystoma tigrinum from a polluted pond (Rose, 1981; Rose & Harshbarger, 1977). Cutaneous papillomas have been experimentally induced in lizards, Lacerta agilis with dimethyl benzantracene (Stolk, 1963).

Chemical contaminants may also play a role in the pathogenesis of certain neoplastic diseases by disrupting immune functions that would otherwise allow the host to eliminate transformed cells. The effects of various immunotoxins have been reviewed by Dean et al. (1990). Chemical effects on immune function in fish has also been

reviewed (Anderson et al., 1984; Dunier, 1994; Zeeman & Brindley, 1981). Associations between chemical contaminants and immune dysfunction have been shown (Lahvis et al., 1995) and experimentally demonstrated in some marine organisms (Arkoosh et al., 1994; DeSwart et al., 1994). Contaminants may also disrupt the immune system indirectly by disrupting neuroendocrine functions (Colborn et al., 1993).

The role of chemical contaminants in green turtle fibropapillomatosis is unknown. As described previously, there is a possible association between high GTFP prevalence and near-shore marine habitats that have been impacted by human activity (Table 2-1). Although it is possible that environmental degradation and contaminants play a role in disease expression, more objective documentation of these impacts in high and low GTFP prevalence sites are needed.

Problems arise in how to document the contaminant exposure of marine turtles. Few data are available for comparing contaminant residue levels in water, sediment, or benthic organisms from high GTFP prevalence areas with those from areas where GTFP is rare. Similarly, data on contaminant levels in green turtle tissues are scant and difficult to obtain because of the endangered status of this species (Aguirre et al., 1994a; Clark & Krynnitsky, 1980; Hall et al., 1983; McKim & Johnson, 1983; Rybitski, 1993; Thompson et al., 1974). The few studies that have been published are difficult to interpret in the context of GTFP. For example, while one

study in 1983 found significant amounts of hydrocarbons in 2 green turtles that stranded after a major oil spill (Hall et al., 1983), most surveys of organochlorine and polychlorinated biphenyl residues in green turtle tissues including egg (Aguirre et al., 1994a; Clark & Krynitsky, 1980; McKim & Johnson, 1983; Rybitski, 1993; Thompson et al., 1974) have yielded relatively low levels, often below the limits of detection of the methods.

Where data exist, there are problems with relating contaminant levels to disease prevalence. First of all, the biologic effect (toxicity) of any particular residue level in green turtles is unknown. Second, surveys of residue levels are usually limited to those chemicals that persist in the environment or bioaccumulate, although important toxic effects such as genetic damage (in a multistage carcinogenesis model) can result from transient exposures to compounds that do not bioaccumulate. In addition, exposure to a potent chemical carcinogen may occur transiently in a completely different habitat from that being monitored. Third, toxic effects may not be direct as in some experimental models, but may involve complex interactions with other abiotic and biotic factors. Thus, fulfilling the criteria for implicating chemical contaminants as the primary cause of GTFP or as cofactor could be extremely difficult (Foster et al., 1993; Hanson, 1988). Finally, the same biological effects may be caused by any number of different

compounds acting through several different mechanisms. Decisions about which contaminant residues to measure should be made with specific a priori mechanistic hypotheses in mind or in light of documented history of exposure to specific compounds.

Nevertheless, there is a need to conduct further toxicological studies. Specifically, there is a need to collect data on the water, sediment, and turtle tissue levels of several classes of chemical contaminants (including known chemical carcinogens and immunotoxins) from several carefully matched marine sites with different prevalences of disease. In addition, controlled experiments involving exposure of turtles to water or sediment extracts from high and low prevalence areas will be necessary in order to clearly demonstrate a contaminant effect in the etiology and pathogenesis of this disease.

Infectious Diseases

The epizootiologic patterns observed among free-ranging green turtle populations including the sudden appearance of GTFP at new geographic sites, variation in prevalence over relatively short distances, and temporal variation within a locality are compatible with an infectious etiology. The observation that some animals recover from GTFP is also compatible with an infectious disease. In addition, an infectious agent is the most plausible explanation for the appearance and spread of GTFP among captive green turtles.

For example, the outbreak documented at Cayman Turtle Farm, Grand Cayman, in 1980, began in wild caught adults and subsequently developed in captive reared turtles over several years. Once eliminated, GTFP has not recurred at Cayman Turtle Farm despite little change in husbandry conditions (Jacobson, 1981b; Jacobson et al., 1989). A similar outbreak occurred in a head start facility in the Florida keys among 2 year old captive reared green turtles that had been held in a pond where GTFP affected turtles were rehabilitated and possibly had direct contact with affected turtles (Hoffman & Wells, 1991).

Viruses

A number of virus families (Papovaviridae, Herpesviridae, Adenoviridae, Poxviridae, Retroviridae) are known to induce proliferative and or neoplastic lesions. Papillomaviruses (Papovaviridae) are the documented cause of papillomas, fibromas, and fibropapillomas in many mammalian and avian species (Sundberg, 1987) and are associated with malignant neoplasia as well (Sundberg & O'Banion, 1989; Zur Hausen, 1989). Among reptiles, a papillomavirus has been described from hyperplastic skin lesions of 5 Bolivian side-necked turtles, Platemys platycephala (Jacobson et al., 1982), and papovavirus-like particles have been observed in papillomas of green lizards, Lacerta viridis (Cooper et al., 1982; Raynaud & Adrian, 1976). A polyomavirus (Papovaviridae) of hamsters produces benign cutaneous neoplasia in these

rodents (Graffi et al., 1968) but other polyomaviruses of rodents and primates do not produce disease in their natural hosts (Eckhart, 1990). Herpesviruses have been associated with cutaneous papillomas and or fibromas in green lizards, Lacerta viridis (Raynaud & Adrian, 1976), african elephants, Loxodonta africana (Jacobson et al., 1986b), carp, Cyprinus carpio (Hedrick et al 1990; Sano et al., 1985), and several salmonids (Kimura et al., 1981a, 1981b, 1981c; Sano et al., 1983; Yoshimizu et al., 1987). Poxviruses are responsible for fibroepithelial proliferative lesions in squirrels (Hirth et al., 1969; O'Connor et al., 1980), rabbits (Pulley & Shively, 1973; Shope, 1932), and primates (Behbehani et al., 1968). Retroviruses have been associated with or proven to be the cause of dermal sarcomas in walleyes, Stizostedion vitreum (Martineau et al., 1991), lip fibromas in angelfish (Francis-Floyd et al., 1993), neurofibromas in damselfish, Pomacentrus partitus (Schmale & Hensley, 1988), myxofibromas and rhabdomyosarcomas of snakes (Lunger et al., 1974; Zeigel & Clark, 1969), sarcomas in poultry (Benjamin & Vogt, 1990), and fibromas and sarcomas in a variety of mammalian species including cats (Hardy, 1985; Moulton & Harvey, 1990) and primates (Gardner & Marx, 1985; Tsai et al., 1990). The molecular mechanisms of virus induced proliferation and oncogenesis vary, but all in some way disrupt the cells' signal transduction network (Benjamin & Vogt, 1990; Moran, 1993; Zur Hausen, 1991). Examples include, the E5 protein of

certain papillomaviruses that binds and activates the PDGF receptor (Kulke & DiMaio, 1991; Petti & DiMaio, 1992; Petti et al., 1991), the adenovirus E1B protein and papillomavirus E6 protein that bind and inactivate cell cycle checkpoint protein p53 (Benjamin & Vogt, 1990; Moran, 1993), and adenovirus E1A, polyomavirus T antigen, and papillomavirus E7 proteins that target the cell cycle control protein pRB. Certain viruses produce proteins that act like growth factors or their receptors. For example, poxviruses may produce epidermal growth factor (EGF)-like peptides (Brown et al., 1985), simian sarcoma virus (a retrovirus) produces a PDGF-like peptide (Benjamin & Vogt, 1990), and certain herpesviruses express a protein with protein kinase activity similar to receptor kinases (Smith et al., 1992).

Evidence for viruses. Certain histologic features of GTFP, including perivascular lymphocytic infiltrates, vesicle formation, and epithelial degeneration are consistent with, although not specific for, virus infection, and have prompted investigators to search for viruses. Smith and Coates (1938) failed to find virus-like inclusions within the tumors that they examined. Jacobson et al. (1989) examined tumors from six turtles from Florida and one turtle from Hawaii by light and electron microscopy. In some sections, cells in the stratum spinosum and outer layers of the epidermis were hypertrophic and vacuolated and amphophilic intranuclear inclusion bodies suggestive of herpesvirus infection were

occasionally seen. Ultrastructural examination revealed mild acanthosis (3-6 cells thick) and intracytoplasmic vacuoles containing 150-170 nm diameter granules of varying electron densities were described within the superficial epidermis but not identified (Jacobson et al., 1989). Aguirre et al. (1994b) described basophilic intranuclear inclusions in several lesions that they suspected to be nucleoli, but also considered compatible with viral inclusions. However, viral particles were not found within these inclusions when examined by EM. They also observed intracytoplasmic electron dense granules approximately 150 nm in diameter that were morphologically similar to viral particles. These, however, were found in both normal and GTFP epithelium. These intracytoplasmic granules are now generally accepted to be mucin granules that are produced and secreted by normal turtle keratinocytes as they differentiate (Aguirre et al., 1994b; Jacobson, 1989; Matoltsy & Huszar, 1972).

Herpesvirus-like particles were demonstrated by electron microscopy in some fibropapillomas taken from two juvenile green turtles housed in the same rehabilitation facility in the Florida Keys (Jacobson et al., 1991). In 3 fibropapillomas examined from one turtle and 1 of 14 tumors examined from the second turtle, focal areas of ballooning degeneration of superficial epithelium were found to contain eosinophilic intranuclear inclusions. Electron microscopy demonstrated the presence of particles within the nucleus

conforming in size and morphology (icosahedral 77-90 nm diameter) with immature herpesvirus and intracytoplasmic particles conforming with mature enveloped herpesvirus (110-120 nm diameter). This agent was not successfully cultured so experiments to fulfill Koch's postulates could not be conducted.

Immunologic and molecular methods have been used to search specifically for papillomaviruses in GTFP biopsies because of the similarities between turtle fibropapillomas and those of other vertebrates known to be caused by this type of virus (Sundberg, 1987). Jacobson et al. (1989) were unable to demonstrate the presence of papillomavirus group-specific structural antigens in paraffin embedded sections of fibropapillomas from 1 Hawaiian and 6 Florida green turtles using peroxidase-antiperoxidase immunohistochemistry. Total DNA extracted from portions of these same tumors was probed under low stringency conditions with bovine papillomavirus type 2 virion DNA. Finally, a reverse Southern blot was performed in which radiolabelled tumor DNA from two turtles was allowed to hybridize with blots containing 25 different cloned papillomavirus genomes (6 bovine, 7 human, and dog, rabbit, coyote, mouse, rat, and parrot papillomaviruses). These screenings for papillomavirus yielded negative results (Jacobson et al., 1989). Similarly, Marc Van Ranst (Einstein Medical College, Bronx, New York, pers. comm.) had negative results when he performed low stringency southern blot

analysis on DNA extracts of 11 tumors collected from a single immature green turtle from the Florida Keys. Probes included full genomic DNA from human papillomaviruses HPV-1, HPV-2, and HPV-5, bovine papillomavirus BPV-1, canine oral papillomavirus, and pygmy chimpanzee papillomavirus PCPV-1.

Preliminary experiments have also been conducted using the polymerase chain reaction and degenerate PCR primers for conserved sequences in the E1 and L1 mammalian papilloma virus genes. These primers failed to amplify any sequences in 11 GTFP biopsies from one green turtle (Van Ranst, pers. comm.).

These negative immunohistochemical and molecular data are insufficient to rule out a papillomavirus as a potential etiologic agent because papillomaviruses are extremely diverse and it is not unlikely that a reptilian papilloma virus would fail to react with mammalian and avian probes, primers, and antisera (O'Banion et al., 1992).

The possible role of oncogenic retroviruses has never been investigated. As with the papillomaviruses, retroviruses may cause neoplasia without ever developing a patent life cycle in the host (Benjamin & Vogt, 1990; Coffin, 1990). However, in the absence of electron microscopic evidence for virus production and shedding from the tumor it is difficult to implicate a retrovirus as the cause. Detection of integrated retroviral genomes (provirus) within the green turtle genome will require specific molecular probes that

will become available only after the agent has been identified and portions of its genome sequenced. Until then it is unlikely that non-specific retroviral probes would yield conclusive results given the ubiquity of endogenous retroviral sequences in vertebrates (Coffin, 1990).

Bacteria

Chronic bacterial infections may induce proliferative lesions in some tissues. For example, intracellular Campylobacter-like organisms are associated with proliferative enteritis in ferrets, hamsters, and swine (Fox & Lawson, 1988; Lawson et al., 1985). An invasive spirochaete has been observed in papillomatous foot lesions in cattle but experiments to fulfill Koch's postulates have not yet been conducted (Read et al., 1992). Numerous species of bacteria have been cultured from the surfaces of cutaneous green turtle fibropapillomas (Aguirre et al., 1994b). However, bacteria are not seen within intact GTFP lesions, and little inflammation is observed within tumors unless the surface is ulcerated, suggesting that these are all secondary opportunistic pathogens.

Metazoan parasites

An association between parasites and neoplasia has been made in several species. Dogs infected with the nematode, Spirocerca lupi, which encysts in the esophagus, may develop a fibrosarcoma at the site (Bailey, 1963). Rats with tapeworm Cysticercus (Taenia) cysts were reported to develop hepatic

carcinomas with high frequency (Dunning & Curtis, 1946). Schistosoma mansoni infection with egg shedding in the urinary tract has been associated with bladder cancer in humans (Hashem et al., 1961).

Marine turtles are host to a variety of digenetic trematode species (Lauckner, 1985). Benign papillomatous lesions in the gallbladder of green turtles have been associated with flukes and eggs of Rhytidodoides similis (family: Rhytidodidae) (Smith et al., 1941). At least 12 species of spirorchid trematodes have been described in the green turtle (Lauckner, 1985). Their natural history is very similar to Schistosoma in that adult trematodes inhabit the vascular system and eggs must migrate through tissues to reach an outlet to the environment. Schistosoma mansoni egg antigens can elicit a fibrotic response in the host (Lammie et al., 1986; Phillips & Lammie, 1986; Wyler, 1983) and it has been suggested that spirorchid eggs may induce fibromas by similar mechanisms (Harshbarger, 1984).

Evidence for a metazoan parasite etiology. The association of spirorchid egg deposition with fibropapillomatosis was first noted by Smith and Coates (1939), who found eggs of Hapalotrema constrictum in sections of over half of 230 fibropapillomas that they examined from Florida green turtles. Later, Jacobson et al. (1989) did not find ova in any sections of 28 tumor biopsies collected from 6 Florida green turtles but eggs were present in tumor

sections from 1 Hawaiian turtle. Norton et al. (1990) and Jacobson et al. (1991) found eggs in many sections of tumors from 3 Florida turtles. Williams et al. (1994) found eggs in fibropapillomas examined from 39 Caribbean green turtles, and Aguirre et al. (1994b) found eggs in biopsy sections from 8 of 10 Hawaiian turtles affected with GTFP.

On the other hand, spirorchid ova have been found in 16 of 21 (76%) wild green turtles, 3 of 10 (33%) oceanarium-reared green turtles, and 3 of 102 (2.9%) farmed green turtles from Queensland, Australia where the prevalence of fibropapillomatosis was 0% (Glazebrook & Campbell, 1990a, 1990b). In addition, ova and lesions associated with ova have been found within otherwise normal tissues of GTFP affected turtles (Aguirre et al., 1994b; Norton et al., 1990).

The significance of cardiovascular trematodes in the etiology of GTFP remains unclear. On the one hand, some authors have characterized cutaneous fibromas from green turtles as a hyperplastic response to spirorchid eggs (Harshbarger, 1984) although the occurrence of spirorchid ova and ova induced lesions in otherwise normal tissues of turtles with GTFP and in those that do not have GTFP argues against a direct hyperplastic or tumorigenic effect. On the other hand, Smith and Coates (1939) concluded that trematode eggs were incidental, accumulating passively in the microvasculature of tumors. Other authors have also tended to

discount the eggs as the cause of GTFP (Jacobson et al., 1989; Lauckner, 1985).

Finally, an argument has been put forward that ectoparasites may have some role in the pathogenesis of fibropapillomatosis. Nigrelli (1942) and Nigrelli and Smith (1943) found leeches, Ozobranchus branchiatus, infesting the folds of papillomas and suggested that the leeches may act as vectors of the causative agent(s). Most authors agree, however, that leeches do not cause tumors directly, although they may severely debilitate their host (Schwartz, 1974). In addition, hirudin secretion may cause some increased vascularization at leech attachment sites (Lauckner, 1985; Nigrelli & Smith, 1943). Aguirre et al. (1994b) reported finding mites attached to the surface of Hawaiian fibropapillomas but did not speculate on the significance of this association.

Genetic Factors

Neoplastic transformation, by definition, involves permanent, often multiple, changes in the cell's genotype leading to relatively unregulated proliferation and differentiation (Sirica, 1989). Familial patterns of neoplastic disease arise from a heritable (germline) genetic lesion that renders individuals more susceptible to disease development following subsequent somatic cell genetic damage. For example, a germ line loss of function mutation in a tumor suppressor locus would predispose an individual to neoplasia

following any event that disables the remaining functional allele (Haber & Housman, 1991; Knudson, 1986; Marshall, 1991). Familial patterns of neoplasia are well documented in humans. Examples include Li-Fraumeni syndrome, Wilm's tumor, retinoblastoma, and neurofibromatosis (Haber & Housman, 1991; Marshall, 1991). Laboratory mice show extensive strain variation in susceptibility to experimental tumorigenesis (DiGiovanni, 1989). A well documented example of genetic susceptibility to neoplasia among lower vertebrates is found in certain platyfish, Xiphophorus maculatus/Xiphophorus helleri hybrids, which have high rates of spontaneous and ultraviolet light induced melanoma due to loss of a functional tumor suppressor gene (Anders et al., 1984; Friend, 1993; Sobel et al., 1975). Heritable defects in DNA repair mechanisms could also render individuals more prone to neoplasia as is the case in xeroderma pigmentosa (Dresler, 1989; Kraemer et al., 1984). The familial pattern of epidermodysplasia verruciformis is believed to involve heritable defects in cellular immune function and an inability to eliminate papillomavirus infection (Orth, 1987; Shah & Howley, 1990). In rabbits, certain major histocompatibility loci have been associated with the regression or progression to malignancy of Shope papilloma induced tumors (Han et al., 1992). In addition, individuals of some species have genetic predispositions to exuberant hyperplastic responses to wounding, e.g. keloidosis in humans

(Caro & Bronstein, 1985) and "proud flesh" in horses (Smith et al., 1972), that can resemble benign neoplasia.

The possibility that some green turtles have a genetic predisposition to develop GTFP must be considered. However, there is no evidence that this is the case because genealogical studies in this species are impractical and methods to distinguish susceptible from resistant individuals are unavailable.

Immune Dysfunction in GTFP Pathogenesis

The possibility that various biological and environmental agents may be involved indirectly in GTFP by causing immune system dysfunction has been alluded to in each of the preceding sections. While immune suppression would not be a necessary prerequisite for infection, if GTFP were caused by a primary infectious agent, the disease would probably be more persistent and severe (Chretien et al., 1978; Duncan et al., 1975; McMichael 1967) and more likely to progress to malignancy (Schneider et al., 1983) in those individuals with compromised cellular immune function.

Implicating immune system dysfunction in the pathogenesis of GTFP will be difficult because few turtle specific reagents are available, immune function assays have not been validated, and normal reference ranges have not been established for green turtles. Immune system studies in sea turtles are in their infancy. Collins (1983) provided initial anatomic descriptions of green turtle lymphoid tissues. The

immunoglobulin classes of green turtles have been described (Benedict & Pollard, 1972, 1977) and some preliminary investigations of cellular immune functions have been conducted (McKinney & Bentley, 1985). Matters are complicated by the fact that the turtle immune system, as in other poikilotherms, is influenced by both season and temperature (Ambrosius, 1976; El Ridi et al., 1988; Muthukkaruppan et al., 1982; Zapata et al., 1992). Eventually, systematic surveys that compare immune function parameters among apparently healthy turtles from populations with high and low GTFP prevalences will be needed to test the hypothesis that immune system dysfunction renders some green turtle populations more susceptible to GTFP. If evidence for an association between immune dysfunction and GTFP is found, it will become important to identify those factors responsible for immunomodulation in affected populations.

Conclusion

This chapter has brought together the available published information about green turtle fibropapillomatosis and provides the background for developing testable hypotheses addressed in the following chapters of this dissertation. It is clear from this review that the major question to be addressed is, what is the etiology of GTFP? Once the nature and identity of the etiologic agent are known one can begin to develop strategies for monitoring and preventing the spread of GTFP among turtle populations. With

techniques to monitor populations for exposure to the causative agent(s) one can begin to model the long-term demographic effects of this disease and initiate studies designed to identify those factors that have allowed this disease to become a worldwide epizootic.

CHAPTER 3
DEVELOPMENT OF MONOCLONAL ANTIBODIES FOR THE DETECTION OF
CLASS-SPECIFIC IMMUNE RESPONSES IN THE GREEN TURTLE

Introduction

There are 7 extant species of marine turtles, all of which are threatened or endangered due to a variety of factors such as over-harvesting, loss of nesting and feeding habitats, marine pollution, and entanglement (National Research Council, 1990). The impact of disease and the relationship of disease susceptibility to environmental factors are poorly understood, due in part to the lack of diagnostic reagents with which to monitor the health status of sea turtle populations. The importance of improved health monitoring capabilities in wildlife conservation is becoming increasingly recognized (Klein, 1993). The urgent need to develop diagnostic tests for green turtles, Chelonia mydas, stems in part from recent worldwide increases in the prevalence and severity of green turtle fibropapillomatosis and the need to better understand the epizootiology of this disease (Chapter 2).

The development of standardized serodiagnostic tests for green turtles would be facilitated by the availability of monoclonal antibodies (Mabs) to specific turtle immunoglobulin classes. Monoclonal antibodies are highly

specific and uniform reagents with reliable performance characteristics that can be obtained in potentially unlimited quantities. This paper describes the production and validation of a battery of monoclonal antibodies specific for each of the known immunoglobulin classes of the green turtle (Benedict & Pollard, 1972).

Materials and Methods

Turtle Plasma Samples and Turtle Immunizations

Blood samples were collected into lithium heparin tubes from the dorsal cervical sinus (Owens & Ruiz, 1980) of 4 green turtles housed in a rehabilitation facility in Marathon, Florida. The plasma obtained from these samples was used to prepare immunoglobulins for use as antigen in hybridoma production. Two juvenile captive-reared green turtles housed at Cayman Turtle Farm, Grand Cayman, British West Indies were immunized by biweekly subcutaneous inoculations with 250 μ g 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) (Molecular Probes, Eugene, OR, USA) in Ribí's adjuvant (RIBI ImmunoChem Research, Hamilton, MT, USA) for a total of 6 inoculations, followed by monthly inoculations of the same DNP-BSA dose for another 8 months. A pre-immunization blood sample was collected, followed by biweekly test bleedings between each of the first 6 inoculations, and monthly bleedings before each monthly booster inoculation. These plasma samples were used to assess the ability of Mabs to measure specific turtle anti-DNP responses and for affinity

purification of anti-DNP antibodies. In addition, pooled plasma samples from loggerhead (Caretta caretta), olive ridley (Lepidochelys olivacea), Kemp's ridley (Lepidochelys kempii), hawksbill (Eretmochelys imbricata), and leatherback (Dermochelys coriacea) were obtained from various sources for testing cross-species reactivity of the Mabs.

Preparation of Turtle Immunoglobulins

Several strategies were employed to isolate and purify turtle immunoglobulins for use in mouse immunizations and hybridoma screening. Initially, putative immunoglobulins were identified and isolated according to their physico-chemical properties. Later, additional approaches were taken, as reagents and antigen specific antibodies from specifically immunized turtles became available.

Globulins from a 50 ml sample of plasma from an individual green turtle and from a 100 ml pooled sample from 3 green turtles from Marathon, Florida were precipitated with saturated ammonium sulphate (SAS) (33% v/v). The precipitate was resuspended in PBS/az (0.01 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.02% NaN₃) and the precipitation repeated. The precipitate was dialyzed into either PBS/az or 0.01 M Tris-HCl buffer (pH 8.0) and adjusted to a final protein concentration of 2 mg/ml.

One portion (5 ml) of the globulin preparation (33% SAS cut) in Tris buffer was applied to a diethylaminoethyl (DEAE) anion exchange column and eluted in steps with 0.01 M Tris

buffer containing either 0.125 M NaCl, 0.25 M NaCl, 0.5 M NaCl, or 1.0 M NaCl.

Another portion (18 ml) of the globulin preparation in PBS/az was applied in 6 ml sample amounts to a 2.5 x 100 cm Sephacryl S-300 column in order to separate proteins on the basis of their size. Fractions were eluted with PBS/az at a 30 ml/hr flow rate and collected using a Gilson fraction collector. Selected eluted protein fractions were reduced by boiling for 5 minutes in Laemmli sample buffer (Laemmli, 1970) with 2-mercaptoethanol, and examined by SDS polyacrilamide gel electrophoresis (SDS-PAGE) using a Phastgel apparatus (Pharmacia LKB, Uppsala, Sweden). Fractions containing similar protein composition were pooled and concentrated in centrifuge filter concentrators (Amicon Centriprep^R-10, W.R. Grace & Co, Beverly, MA, USA). Selected DEAE fractions were used to immunize mice and the gel filtration fraction pools were used as antigen in preliminary hybridoma screening protocols.

Immunoglobulin purification by anti-light chain affinity column chromatography. An affinity column was prepared using 2 mg monoclonal antibody HL673 which is specific for the immunoglobulin light chain of the desert tortoise (Schumacher et al., 1993). Mab HL673 which had been found to cross-react strongly with putative light chain of the green turtle in ELISA and Western Blots was covalently linked to a hydrazide support gel (Affi-prep^R Hz, Bio-Rad Laboratories, Richmond,

CA, USA) following manufacturers instructions. Briefly, 1 ml of purified Mab HL673 (2 mg/ml) was oxidized with 20 μ l of sodium periodate stock solution (0.5 M NaIO₄) in oxidation buffer (0.02 M sodium acetate, 0.15 M NaCl, pH 5.0) for 45 minutes at room temperature. The oxidation reaction was stopped by addition of 5 μ l glycerol. The oxidized antibody was dialyzed into coupling buffer (0.1 M sodium acetate, 1.0 M NaCl, pH 4.5) and incubated overnight with approximately 2 ml of settled hydrazide support beads. The antibody coupled beads were then washed with 0.5 M NaCl, 0.01 M phosphate buffer, pH 7.5 and stored at 4°C. The column was prepared, conditioned with elution buffer (0.1 M glycine, pH 2.7), washed with PBS/az, and then 1 ml (2 mg) of green turtle immunoglobulin rich preparation (33% SAS cut) was applied. After washing the column the bound protein was eluted with 0.1 M glycine, pH 2.7. Fractions (1 ml) were collected and neutralized with 45 μ l of 1.0 M Tris, pH 9.0. Eluted proteins were concentrated and examined with SDS-PAGE. These proteins were also used to immunize mice for hybridoma production.

Purification of anti-DNP antibodies by affinity column chromatography. Turtle anti-DNP antibodies were purified using affinity chromatography (Goetzl & Metzger, 1970; Wofsy & Burr, 1969). N e-2,4-DNP-lysine (Sigma Chemical Co, St. Louis, MO, USA) (2 mM in 0.1 M NaHCO₃, pH 8.3) was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia LKB,

Uppsala, Sweden). The DNP-lysine coupled Sepharose was packed into a column 2.5 x 13 cm (37 ml) and washed with 50 ml methanol and then equilibrated in borate buffer (0.015 M NaBO_3 , 0.15 M NaCl, pH 8.0). The column was further washed with 1% bovine serum albumin (in PBS/az) and 25% acetic acid followed by equilibration in high salt borate buffer (0.015 M NaBO_3 , 0.5 M NaCl, pH 8.0) before use. Pooled plasma from the two DNP-BSA immunized turtles was diluted 1:3 in high salt borate buffer and applied to the column. The column was washed until the optical density ($\text{OD}_{280\text{nm}}$) returned to baseline, and then any bound turtle anti-DNP antibodies were eluted with 5 ml 0.1 M 2,4 DNP-glycine (pH 8.6). The eluted fractions were pooled and concentrated to a 2 ml volume and then extensively dialyzed against PBS/az. This solution, containing highly purified turtle anti-DNP antibodies, as judged by ELISA and SDS-PAGE, was used for final screening of newly developed monoclonal antibodies. A small aliquot was dialyzed against 50 mM Tris (pH 7.4) for mass spectrophotometry.

Mass spectrometry. Affinity purified turtle anti-DNP antibodies were submitted to the Protein Analysis Core, Interdisciplinary Center for Biotechnology Research, University of Florida for mass spectrometry (Vestec VT 2000, Perseptive Biosystems/Vestec Mass Spectrometry Products, Houston, TX, USA).

Hybridoma Production

Mouse immunization protocols. One 6-8 week old female BALB/c mouse was immunized subcutaneously with 6 μ g of HL673 affinity purified turtle immunoglobulin in Ribí's adjuvant. Booster immunizations were repeated in two and four weeks. The final booster was 17 μ g of antigen intraperitoneally. Fusion was performed 4 days after the last inoculation. Two 6-8 week old female BALB/c mice were immunized with a DEAE fraction (50 μ g total protein) containing both 5.7S and 7S green turtle immunoglobulins (see results) in Ribí's adjuvant at several subcutaneous sites. Booster immunizations were performed at 2 weeks, 4 weeks (50 μ g antigen per mouse). Immunizations of both mice (100 μ g antigen each) were continued at 2 to 4 week intervals for a total of 7 immunizations using a combination of the 5.7S and 7S IgY rich DEAE fraction (25-75 μ g) and various IgM-rich preparations derived from DEAE and Sephacryl S-300 chromatography runs (45-100 μ g). The two mice differed only in the last immunization. One mouse was rested for about 4 weeks before its final booster with both IgY and IgM whereas the second mouse was rested for 10 weeks before its final booster with turtle IgM alone. Serum anti-turtle titers were checked periodically by ELISA.

Fusions. Monoclonal antibody production followed the standard protocol of the Hybridoma Core Laboratory, Interdisciplinary Center for Biotechnology Research,

University of Florida (Liddell & Cryer, 1991; Simrell & Klein, 1979). Three independent fusions (one for each mouse) were carried out. In general, four days following the final booster immunization, mice were euthanized under methoxyflurane anesthesia and their spleens removed. Splenocytes were prepared by mechanical disaggregation, washed, and fused with log phase SP2/0 mouse myeloma cells in a 7:1 ratio using 50% polyethylene glycol 1500 media (Boehringer Mannheim, Germany). After pelleting by centrifugation at 400 x g for 8 minutes, cells were resuspended in fusion media (D-MEM plus 1 x Antibiotic-Antimycotic, 1 x HAT, 25% SP2/0 Conditioned Media, 20% Horse Serum) (GIBCO, Grand Island, NY, USA) and seeded into 96 well culture plates (Costar, Cambridge, MA, USA). Wells were monitored microscopically for growth of hybridomas.

Screening was begun on growth positive wells 10-14 days post fusion. The supernatants were removed and tested for antibody reactivity against specific antigens (see below). Hybridoma cultures of interest were transferred to 24 well plates and expanded until they could be retested (about 7 days). Hybridoma cultures of interest were safeguarded by cryopreservation in liquid nitrogen. Selected cultures were isotyped using an isotyping kit (Amersham Mouse Monoclonal Antibody Isotyping Kit, Code RPN.29, Amersham, UK) and cloned by limiting dilution.

Monoclonal Antibody Screening Protocols

Hybridoma culture supernatants were screened against each of the three turtle immunoglobulin rich pools derived from the S-300 gel filtration column using enzyme linked immunosorbent assays (ELISA). Secondary screening was done by Western Blotting.

ELISA protocol. A standard ELISA protocol was used for screening (Schumacher et al., 1993). Each well of a microtiter plate (Maxisorp F96, NUNC, Kamstrup, Denmark) was coated with 50 μ l of antigen at a concentration of 10 μ g/ml in PBS/Az and incubated at 4°C overnight. The wells were washed four times with PBS/Az containing 0.05% Tween-20 (PBS-Tween) by an automatic ELISA washer (EAW II, LT-Laboratories, Salzburg, Austria) and then blocked with 300 μ l/well of 1% BSA in PBS/Az at room temperature for 60 minutes or at 4°C overnight. After four more washes, 50 μ l of hybridoma culture supernatant was added to individual wells and incubated at room temperature for 60 minutes. The wells were washed again and 50 μ l of a 1:1000 dilution of alkaline phosphatase conjugated rabbit anti-mouse IgG whole molecule (Sigma Chemical Co, St. Louis, MO, USA) was added to each well. Following incubation at room temperature for 60 minutes, the plates were washed 4 times with PBS-Tween and 100 μ l of p-nitrophenyl phosphate disodium (Sigma Chemical Co, St. Louis, MO, USA) (1 mg/ml prepared in 0.01 M sodium bicarbonate buffer, pH 9.6 containing 2 mM $MgCl_2$) was added to each well

and incubated in the dark at room temperature for 90 minutes. The optical density of each well at a wavelength of 405 nm was measured in an ELISA plate reader (EAR 400 AT, SLT-Laboratories, Salzburg, Austria) at 30, 60, and 90 minutes. Positive and negative controls included on each assay plate consisted of immune mouse serum and hybridoma cell culture medium, respectively. Preliminary screens used selected gel filtration fraction pools (either IgM-rich, 5.7S-rich, or 7S-rich) as antigen (Figure 1). Later ELISA screens used affinity purified turtle anti-DNP antibodies (2 µg/ml) as antigen.

Immunoblotting (Western blotting). Immunoblotting was performed to help demonstrate the specificity of our monoclonal antibodies for immunoglobulin chains. Immunoblots were prepared following a published basic protocol (Schumacher et al., 1993). Briefly, 100-150 µg of green turtle globulins (33% SAS cut) were separated by SDS-PAGE under reducing conditions, using a precast 10% Tris-glycine gel (Novex, San Diego, CA, USA) as previously described (Laemmli, 1970). The proteins were then electrophoretically transferred from the gel to a nitrocellulose sheet (Schleicher & Schuell, Keene, NH, USA) using a transfer apparatus (Novex, San Diego, CA, USA). A Tris-glycine buffer (pH 8.3) in 20% methanol was used as transfer buffer. Blotting time was 120 minutes at 30 volts. Once the transfer was complete, the nitrocellulose was blocked immediately with 5% nonfat dry

milk in PBS/Az and incubated at room temperature on a rocker overnight. The membrane was then washed three times (5 minutes per wash) with PBS-Tween and placed into a trough-manifold (PR 150 Mini Deca Probe, Hoeffer Scientific Instruments, San Francisco, CA, USA). Hybridoma culture supernatants were loaded, 300 μ l per channel, and incubated on the nitrocellulose for 90 minutes at room temperature on a rocker. The nitrocellulose membrane was washed 3 more times and then incubated with 300 μ l of a 1:1000 dilution of alkaline phosphatase conjugated rabbit anti-mouse IgG whole molecule for 90 minutes at room temperature. The membrane was then removed from the manifold, washed 3 times and developed with substrate buffer (0.1 M Tris-HCl, 1 mM MgCl₂, pH 8.8) containing 44 μ l of nitroblue tetrazolium chloride (NBT) and 33 μ l of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) per 10 ml of substrate buffer (Immunoselect, GIBCO BRL, Gaithersburg, MD, USA). Immunoblots using biotinylated Mabs followed the same basic procedure except that biotinylated Mabs diluted in 1% BSA-PBS/az to 1 μ g/ml replaced hybridoma culture supernatants and strepavidin-alkaline phosphatase (Zymed Laboratories, San Francisco, CA, USA) replaced the alkaline phosphatase conjugated rabbit anti-mouse.

Monoclonal Antibody Purification and Biotinylation

Selected cloned hybridoma lines were injected i.p. into Pristane-primed BALB/c mice and the resulting ascites fluid

containing the desired monoclonal antibodies was harvested. Monoclonal antibodies were purified from ascites by passage over a Protein G Sepharose Fast Flow affinity column (Pharmacia LKB, Uppsala, Sweden) and biotinylated.

Each purified Mab was dialyzed against 0.1 M NaHCO₃, pH 8.0 and adjusted to a final concentration of 1.0 mg/ml (Goding, 1986). Sulphosuccinimidyl-6-(biotinamido) hexanoate (Immuno Pure NHS-LC Biotin, Pierce, Rockford, IL, USA) dissolved in dimethyl sulphoxide at 1.0 mg/ml was added (120 µg biotin per mg of antibody) and the mixture was incubated for 2 hours at room temperature. Following incubation, the Mabs were dialyzed into PBS/az and stored at 4°C.

Cross Species Reactivity of Monoclonal Antibodies

Supernatants from hybridomas producing Mabs specific for green turtle immunoglobulins were screened by ELISA for reactions with 33% SAS globulin preparations of 5 other sea turtle species. The ELISA followed the general procedure but used each 33% SAS cut at 5 µg/ml coating concentration as antigen.

Verification of Monoclonal Antibody and Turtle Antibody Specificity

The following experiments were conducted to prove further that developed Mabs were specific for individual turtle immunoglobulin classes and would react with turtle antibodies.

Sandwich ELISA protocol. An antigen capture experiment was designed to test whether the turtle plasma proteins bound by each Mab possessed an immunoglobulin light chain. ELISA plates were coated with 50 μ l per well of selected purified monoclonal antibody (5 μ g/ml). Following incubation with green turtle 33% SAS cut (2 μ g/ml), the sandwich was completed with 1 μ g/ml biotinylated HL673 (anti-light chain) and detected with strepavidin-alkaline phosphatase.

Detection of immune responses to DNP-BSA. Biotinylated Mabs were used in an ELISA format to measure anti-DNP antibody responses in turtles immunized with DNP-BSA. The general ELISA protocol (described above) was used except that Polysorp plates (Polysorp, NUNC, Kamstrup, Denmark) were coated with 50 μ l per well DNP-BSA (1 μ g/ml) and blocked with 2.5% casein (pH 7.0). Plasma samples from DNP-BSA immunized turtles were diluted 1:50 in 1% BSA-PBS/az and serial two-fold dilutions were tested. Plates were incubated with class specific biotinylated monoclonal anti-turtle antibody (1 μ g/ml in 1% BSA-PBS/az) followed by strepavidin-alkaline phosphatase (Zymed Laboratories, San Fransisco, CA, USA).

Competitive inhibition assays. Competitive inhibition ELISA's were used to verify that the turtle plasma proteins, i.e. antibodies, detected by each Mab were DNP-specific. First, plasma samples with peak anti-DNP responses from the 2 immunized turtles were serially diluted and incubated at 4°C overnight with increasing concentrations of soluble hapten

(2,4 DNP-glycine pH 7.4 in PBS/az final concentration range: 0-2 mM). These "inhibited" plasma samples were then assayed for residual antibody activity by ELISA as described above. Second, plasma samples with peak anti-DNP responses were serially diluted and mixed with serial twofold dilutions of rabbit anti-DNP antiserum (Sigma Chemical Co., St. Louis, MO) with specific anti-DNP antibody concentrations ranging from 0 to 7.5 µg/ml. The residual turtle DNP-specific antibody activity was then assayed by ELISA.

Results

Immunoglobulin Purification

Turtle globulins eluted from the DEAE column in two peaks corresponding to 0.125 M NaCl and 0.25 M NaCl. Analysis by reducing SDS-PAGE revealed that the 0.125 M NaCl peak contained three major protein components with approximate molecular weights of 23 kD, 38 kD, and 65 kD, consistent with immunoglobulin light chain, 5.7S heavy, and 7S heavy chains respectively (see Ambrosius, 1976). The 0.25 M peak contained a mixture of proteins of various sizes, including a 70 kD band consistent with IgM heavy chain (data not shown).

Turtle globulins separated on the Sephacryl S-300 column also eluted in two major peaks: an early peak containing putative IgM and a large late peak containing a mixture of 5.7S and 7S IgY (Figure 3-1A). Fractions were analyzed by reducing SDS-PAGE and those with similar protein composition were pooled and the resulting pools were designated as either

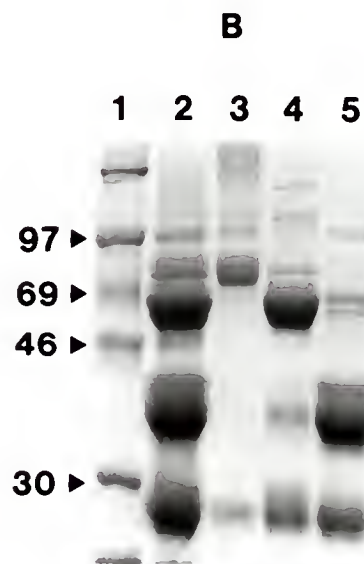
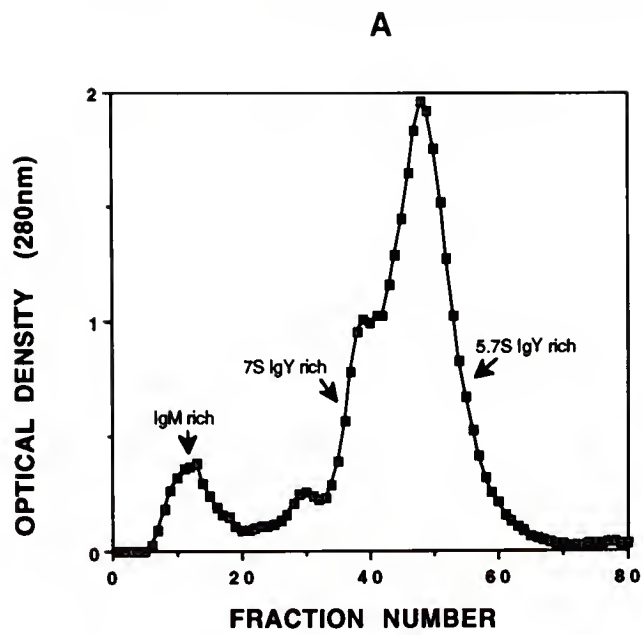
IgM-rich, 5.7S-rich, or 7S-rich (Figure 3-1B). These were used for initial ELISA screening of hybridoma supernatants.

A very small amount of turtle immunoglobulin (< 100 µg) was purified from the globulin preparation with an anti-light chain immunoaffinity column. This material contained three major components of approximately 65 kD, 38 kD, and 23 kD, consistent in size with turtle 7S and 5.7S heavy chains and light chain (data not shown).

Turtle anti-DNP antibodies eluted from the DNP-Sepharose column showed 4 predominant bands on reducing SDS-PAGE. These bands had molecular weights of approximately 23, 38, 65, and 70 kD as expected of the light chain, 5.7S, 7S, and IgM heavy chains respectively (Figure 3-2A). This protein was used in ELISA and western blotting to screen the monoclonal antibody supernatants and purified biotinylated Mabs. Figure 3-2B shows representative western blot results for these Mabs and HL673 (anti-tortoise light chain) and demonstrates the specificity of each Mab for its target immunoglobulin chain. The control Mab, HL860 (anti-turtle non-immunoglobulin), did not react with the affinity purified turtle anti-DNP antibody preparation.

An aliquot of affinity purified turtle anti-DNP antibodies was examined by mass spectrometry. The mass spectrometer detected two proteins with molecular weights of 120 and 175 kD corresponding to the expected molecular weights of intact 5.7S and 7S Ig respectively (Figure 3-3).

Figure 3-1. Fractionation of green turtle immunoglobulins by gel filtration chromatography. Turtle globulins (33% SAS cut) were applied to a Sephacryl S-300 column and eluted with PBS/az. Fractions with similar protein composition by SDS-PAGE analysis were pooled. Three fraction pools were produced: IgM rich (fractions 8-18), 7S rich (fractions 34-40), and 5.7S rich (fractions 50-62). (A) Elution profile of turtle globulins fractionated on S-300 column. Protein content of each fraction was estimated by spectrometry (OD_{280nm}). (B) Reducing SDS-PAGE (10% Tris-glycine) of green turtle immunoglobulin rich fraction pools, stained with Coomassie Blue. Lane 1--molecular weight markers (kD); lane 2--33% SAS cut; lane 3--IgM rich pool; lane 4--7S IgY rich pool; lane 5--5.7S IgY rich pool.



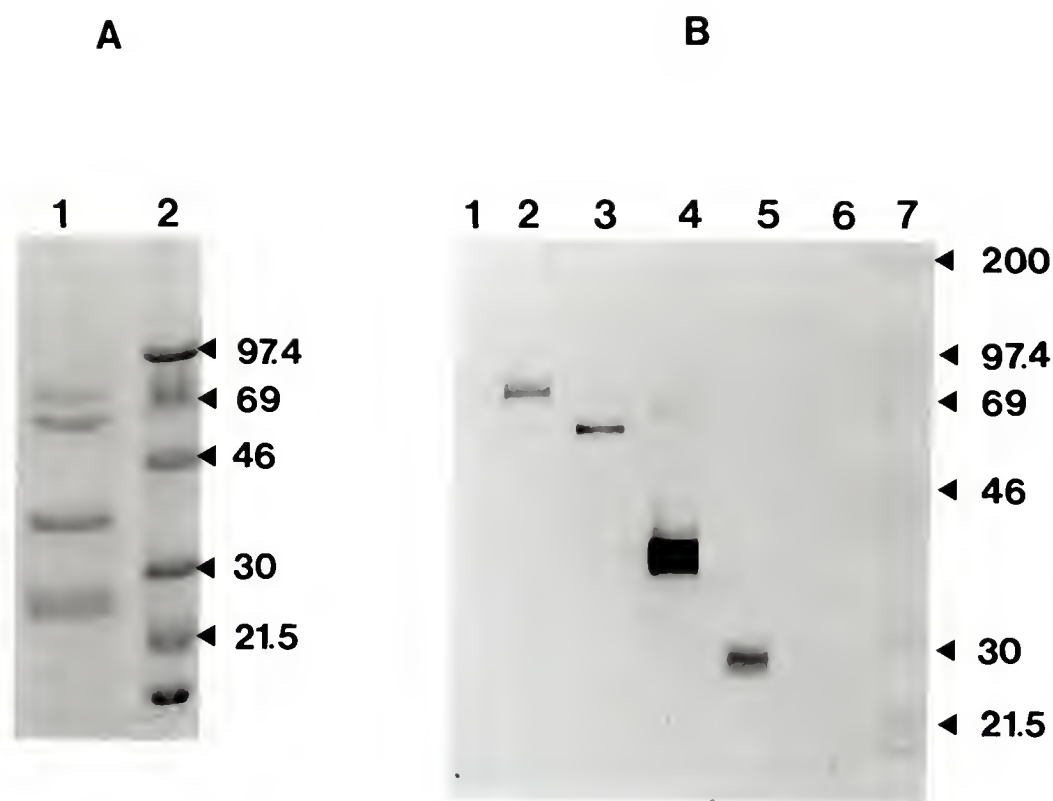


Figure 3-2. Affinity purified turtle anti-DNP antibody chains. (A) Coomassie Blue stained 12% Tris-glycine reducing gel showing turtle anti-DNP antibodies eluted from a DNP-Sepharose affinity column with 0.1 M DNP-glycine. Lane 1--turtle anti-DNP antibodies; lane 2--molecular weight markers. (B) Immunoblot of selected monoclonal antibodies (Mabs) on affinity purified turtle anti-DNP antibodies. Each lane was incubated with a different biotinylated Mab: either HL860 anti-turtle non-immunoglobulin plasma protein (lane 1), HL846 anti IgM (lane 2), HL857 anti-7S IgY heavy chain (lane 3), HL814 anti-5.7S IgY heavy chain (lane 4), or HL673 anti-tortoise IgY light chain (lane 5). Control lane contained 1% BSA (lane 6). Lane 7 contained molecular weight markers.

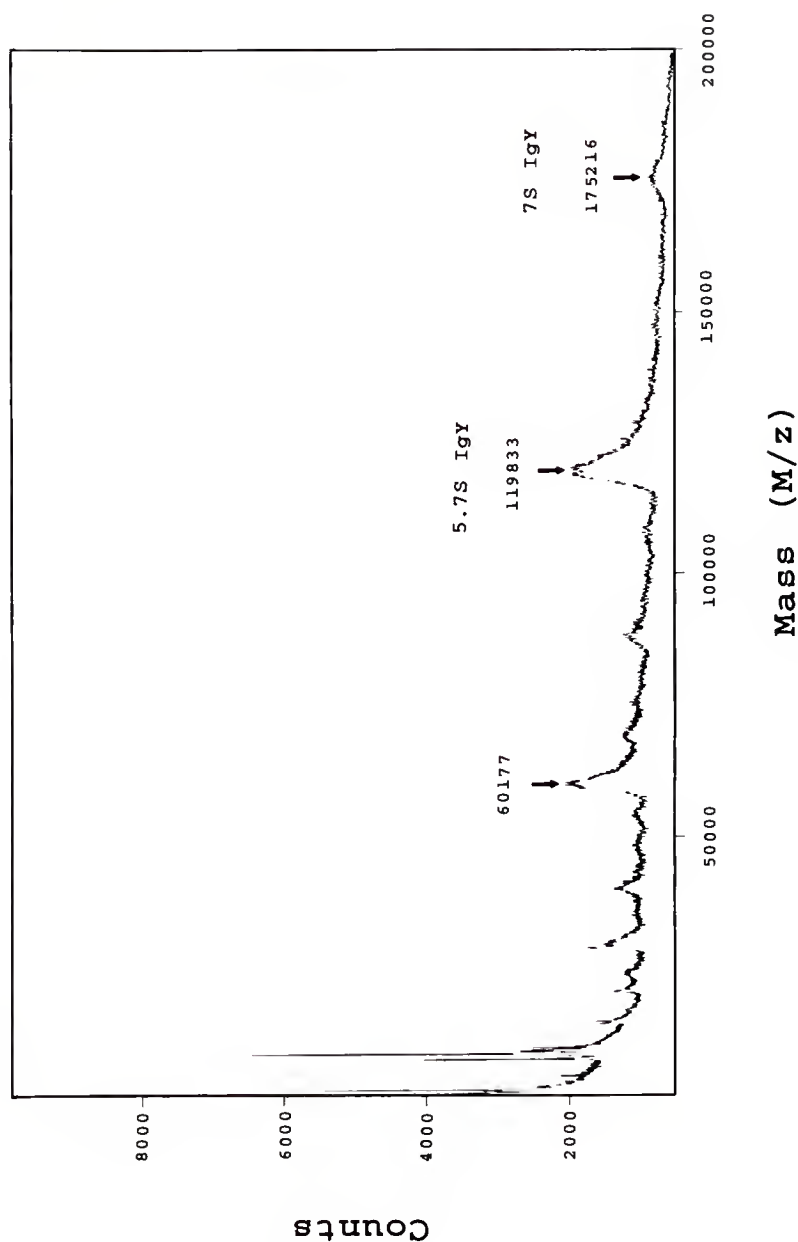


Figure 3-3. Molecular masses of native 5.7S and 7S turtle immunoglobulins. Mass spectrometric analysis (mass to charge ratio) of affinity purified turtle anti-DNP immunoglobulins. Two peaks (arrows) at approximately 120 and 175 kD correspond with expected molecular weights of singly charged 5.7S and 7S proteins respectively. The peak at 60 kD (arrow) could represent either singly charged 7S heavy chain or doubly charged intact 5.7S immunoglobulin. The upper size limit for this analysis is about 200 kD, so native IGM could not be detected.

Native IgM could not be detected by mass spectrophotometry because the size limit for the method is 200 kD.

Production and Characterization of Monoclonal Antibodies

Hybridoma screening by ELISA against various immunoglobulin rich fraction pools and Western blotting against turtle globulins (33% SAS cut) yielded 20 hybridomas of interest which were retained for further study. The initial selection of these hybridomas was based on the specificity of their Mabs for turtle proteins of the appropriate physical and chemical properties. However these results were not sufficient proof that these Mabs were specific for turtle antibodies. Further screening by ELISA against affinity purified turtle anti-DNP antibodies showed that only 15 were specific for turtle immunoglobulin classes. Table 3-1 gives the specificities and isotypes of these 15 Mabs. Ten of these Mabs were specific for 7S IgY heavy chain, whereas 2 Mabs each were specific for the immunoglobulin light chain and IgM heavy chain, and only 1 Mab was specific for the 5.7S IgY heavy chain.

Mabs from the 15 hybridomas that reacted positively with affinity purified turtle anti-DNP antibodies were tested by ELISA against serum globulin fractions (33% SAS cuts) from 5 other sea turtle species. Table 3-1 shows that several monoclonal antibodies reacted with epitopes that are shared broadly among sea turtle species. Nine of the 7s IgY heavy chain specific Mabs cross-reacted with all sea turtle

Table 3-1. List of monoclonal antibodies specific for green turtle immunoglobulins

Hybridoma	Clone	Mab Isotype	Specificity for Turtle Ig ^a	Cross Species Reactivity ^b
92.137.3-1H9	HL814	IgG ₁	5.7S IgY	none
92.137.3-3F6	-	IgG _{2A}	7S IgY	none
92.137.10-3C6	HL857	IgG _{2A}	7S IgY	Cc, Dc, Ei, Lk, Lo
92.137.10-3E11	-	IgG	7S IgY	Cc, Dc, Ei, Lk, Lo
92.137.10-3C3	-	IgG	7S IgY	Cc, Dc, Ei, Lk, Lo
92.137.10-3G11	-	IgG	7S IgY	Cc, Dc, Ei, Lk, Lo
92.137.10-3B5	-	IgG	7S IgY	Cc, Dc, Ei, Lk, Lo
92.137.10-3E10	-	IgG	7S IgY	Cc, Dc, Ei, Lk, Lo
92.137.10-3B11	-	IgG	7S IgY	Cc, Dc, Ei, Lk, Lo
92.137.10-4C3	-	IgG	7S IgY	Cc, Dc, Ei, Lk, Lo
92.137.10-1G10	-	IgG	7S IgY	Cc, Dc, Ei, Lk, Lo
92.137.15-4G11	-	IgG	IgM	none
92.137.15-7A9	HL846	IgG ₁	IgM	none
92.137.15-2C12	-	IgG	Light Chain	Cc, Ei, Lk, Lo
92.137.15-2C11	-	IgG	Light Chain	Cc, Ei, Lk, Lo

^a Confirmed by immunoblot (western blot).

^b Cc = Caretta caretta, Dc = Dermochelys coriacea, Ei = Eretmochelys imbricata, Lk = Lepidochelys kempii, Lo = Lepidochelys olivacea.

species. Both light chain specific Mabs cross-reacted with all species except the leatherback turtle. The IgM and 5.7S Mabs on the other hand seemed to be specific for green turtle only.

Verification of Monoclonal Antibody Specificity

Three hybridomas were cloned and their monoclonal antibodies purified. These were designated HL814 (anti-5.7S IgY heavy chain), HL846 (anti-IgM heavy chain), and HL857 (anti-7S IgY heavy chain) respectively. These Mabs were used for further validation experiments. Purified Mabs against turtle immunoglobulin light chain were not prepared because of the availability of HL673 (anti-desert tortoise light chain).

Sandwich ELISA. An experiment was designed to test whether the turtle plasma proteins being bound by each of these Mabs could be identified as immunoglobulin by the criterion of having an immunoglobulin light chain. Figure 3-4 shows that proteins, selectively captured from turtle plasma SAS cut by Mabs HL814, HL846, or HL857, in turn bound labelled light chain specific Mab HL673, whereas antigen captured by HL860 (Mab specific for an unidentified 33 kD turtle protein present in SAS cut) failed to bind Mab HL673.

Antibody Responses to Immunization. Figure 3-5A-D shows that both HL814 and HL857 could detect rising anti-DNP antibody responses in both experimentally immunized turtles. A rise in 7S IgY anti-DNP activity was detected in both

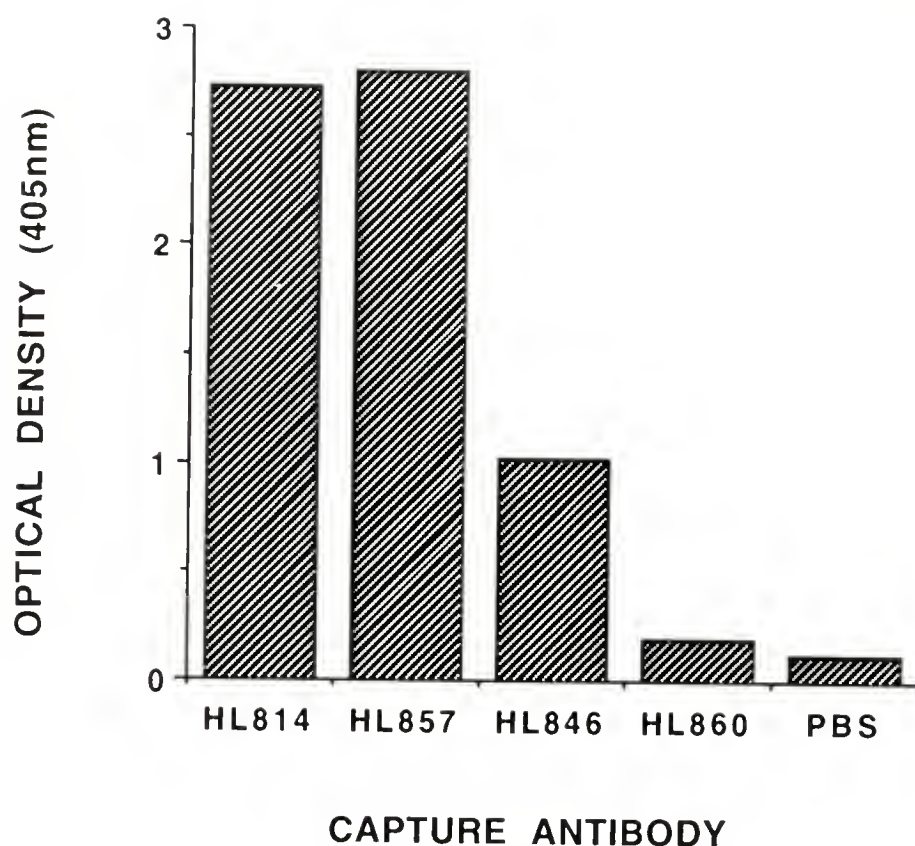


Figure 3-4. Sandwich ELISA demonstrating that putative heavy chain specific Mabs bind plasma proteins with immunoglobulin light chains. ELISA plates were coated with 5 $\mu\text{g/ml}$ of each of the following purified Mabs: HL814 (anti-5.7S heavy chain), HL857 (anti-7S heavy chain), HL846 (anti-IgM heavy chain), and HL860 (anti-non-immunoglobulin plasma protein). Following incubation with 2 $\mu\text{g/ml}$ turtle globulins (33% SAS cut), plates were washed and incubated with 1 $\mu\text{g/ml}$ biotinylated HL673 (anti-tortoise light chain) to complete the sandwich. Binding of HL673 was detected with streptavidin alkaline phosphatase. Data presented are ELISA reactivities ($\text{OD}_{405\text{nm}}$) after 90 minutes incubation with substrate.

turtles within 5 weeks of beginning immunizations and remained high for the remainder of the experiment. The rise in 5.7S IgY anti-DNP activity took up to 9 months to reach a maximum in both turtles. Results for IgM (HL846) were less clear. One turtle (#4624) showed a weak IgM peak at about 13 weeks (Figure 3-5F), whereas the other turtle (#3150) appeared to have a high IgM anti-DNP response in the pre-inoculation sample as well as subsequent samples (Figure 3-5E). Various modifications of the ELISA protocol, such as using high salt (0.5 M NaCl) buffer, failed to reduce the pre-inoculation putative IgM anti-DNP signal. Neither turtle developed detectable antibody titers to BSA after 10 months of immunization with DNP-BSA.

Inhibition by soluble hapten. Figure 3-6 (A-C) shows that the ELISA reactions of immune plasma having peak anti-DNP antibody titers can be inhibited with increasing concentrations of soluble hapten (DNP-glycine). Both the degree of inhibition attained and the shape of the inhibition curves varied between turtles and among antibody classes assayed. Inhibition ranged from 72 to 97% with 1 μ M DNP-glycine. Because no anti-BSA antibody responses could be detected in either turtle, it was impossible to test whether peak anti-BSA responses would be refractory to inhibition by soluble DNP-glycine. Nevertheless, results of this experiment support the conclusion that these Mabs (HL814, HL846, and HL857) recognize DNP-specific antibodies. The ELISA reactions

Figure 3-5. Development of antibody responses to DNP with time in 2 chronically immunized turtles. Serial two-fold dilutions of plasma samples, collected periodically throughout a prolonged immunization schedule (10 months), were tested by ELISA for anti-DNP activity using various biotinylated Mabs. The rise in OD_{405nm} with time is indicative of a rising DNP-specific antibody titer. Turtle 7S IgY (A & B), 5.7S IgY (C & D), and IgM (E & F) responses were detected by biotinylated HL857, HL814, and HL846 respectively. Panels A, C, and E show the responses of turtle #3150 and B, D, and F show those of turtle #4624.

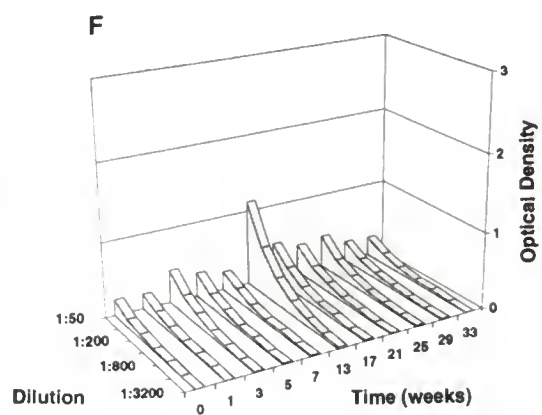
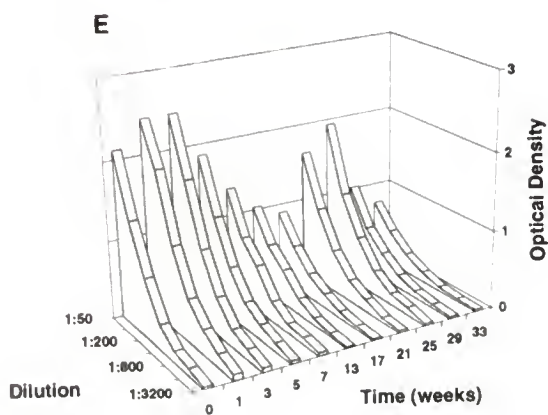
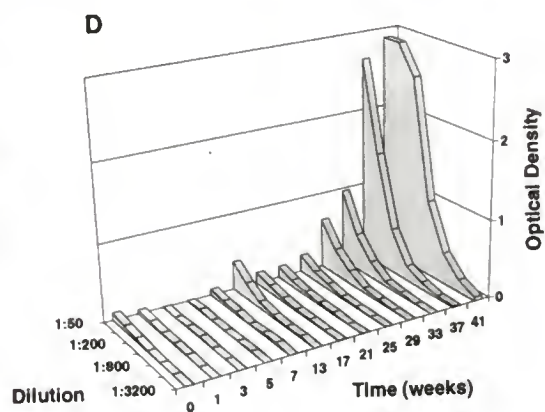
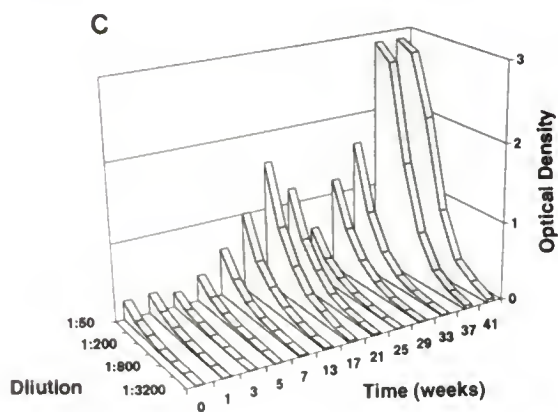
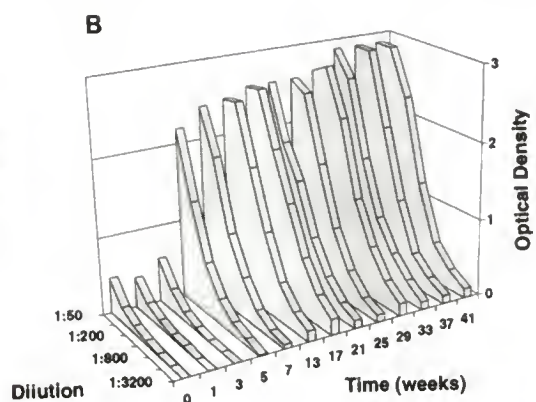
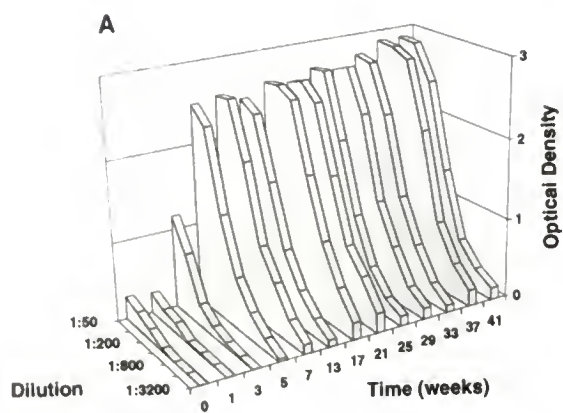
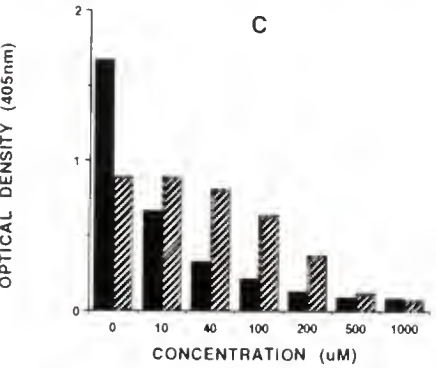
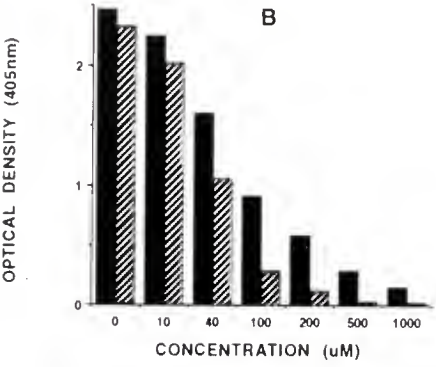
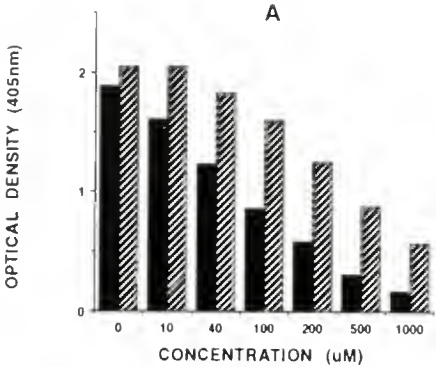


Figure 3-6. Inhibition of turtle anti-DNP antibody activity by soluble antigen. Serially diluted plasma samples with peak 5.7S, 7S, or IgM anti-DNP antibody activity were incubated overnight with final concentrations of 2,4 DNP-glycine ranging from 0 to 1 μ M. Samples were then tested by ELISA for binding to DNP-BSA coated plates. Readings (OD_{405nm}) taken at 60 minutes were plotted against DNP-glycine concentration. The plasma samples used were: week 41 for 5.7S and 7S (both turtles), week 1 for #3150 IgM, and week 13 for #4624 IgM (see Figure 3-5). Data presented are for plasma diluted 1:400 for 5.7S IgY (HL814) and 7S IgY (HL857) and diluted 1:50 for IgM (HL846). Solid bars (turtle #3150); crosshatched bars (turtle #4624).



of immune plasma with peak anti-DNP antibody titers were also strongly inhibited by increasing concentrations of rabbit anti-DNP specific antibodies (data not shown).

Discussion

Sea turtles have 3 major classes of immunoglobulins: a 17 S IgM, a 7S IgY, and a 5.7S IgY (Benedict & Pollard, 1972). IgM is believed to be produced transiently early in an immune response, as in mammals (Benedict & Pollard, 1977; Chartrand et al., 1971). In reptiles, IgM may be the primary immunoglobulin that is secreted onto mucosal surfaces (Portis & Coe, 1975). The 7S IgY is believed to function as a serum antibody like mammalian IgG. The role of 5.7S IgY is unclear, but evidence suggests that it is a chronic immune response globulin and that it is maternally transferred to egg yolk (Benedict & Pollard, 1972, 1977; Chartrand et al., 1971).

The results presented here show that Mabs with specificity for the light chain and each of the three heavy chain classes of the green turtle have been produced. The purification and screening strategies used were dictated in part by the limited availability of turtles and antigen specific plasma. Initially, plasma from specifically immunized green turtles was unavailable, so the preliminary immunoglobulin purification and hybridoma screening relied on identification of plasma proteins with the physico-chemical properties (solubility, size, and charge) consistent with earlier reports on turtle immunoglobulins (Benedict &

Pollard, 1972, 1977; Leslie & Clem, 1972). Previous structural studies of turtle antibodies (several species) indicated that turtle light chains are approximately 22.5 kD and turtle 5.7S IgY, 7S IgY, and IgM heavy chains are 35-38, 63-68 kD, and 70 kD respectively (Ambrosius, 1976; Benedict & Pollard, 1977; Chartrand et al., 1971; Leslie & Clem, 1972). Preliminary screening of fusions yielded a collection of 20 hybridomas that bound to turtle plasma proteins with the appropriate physico-chemical properties. However, additional screenings against antigen-specific turtle antibodies (affinity purified turtle anti-DNP antibodies) revealed that only 15 of these Mabs could be classified as immunoglobulin specific. The turtle proteins recognized by the other 5 Mabs had similar physico-chemical properties but could not be shown to bind antigen.

Additional experiments were conducted to prove further that the selected cloned hybridomas produced Mabs that were specific for turtle antibodies. A sandwich ELISA, using an anti-light chain Mab demonstrated that the turtle plasma proteins recognized by each of the heavy chain specific Mabs possessed a light chain, thereby confirming the specificity of these Mabs for turtle immunoglobulins. Mabs specific for turtle immunoglobulin classes should be able to detect an increasing antibody titer in response to immunization with specific antigen. Mabs HL814 and HL857 (5.7S IgY heavy and 7S IgY heavy chain specific, respectively) were able to measure

an increasing anti-DNP response in two chronically immunized turtles. The 7S anti-DNP response rose rapidly within 5 weeks whereas the 5.7S response required months to develop. Qualitatively, this pattern is consistent with previously published descriptions of the ontogeny of the immune response in green turtles (Benedict & Pollard, 1977).

The IgM heavy chain specific Mab HL846 demonstrated a weak transient response in one animal but not in the other. The IgM peak occurred approximately 2 months after the 7S response developed, which was unexpected. Other workers have had difficulty in consistently demonstrating the classical mammalian type IgM response in green turtles (Benedict & Pollard, 1977), although it has been demonstrated in other turtle species (Chartrand et al., 1971). The type of antigen, adjuvant, dosage, dosing frequency, and testing frequency may influence whether an IgM response occurs and is detected. Previous exposure to antigen will also effect the development of an IgM response. The second turtle appeared to have a high anti-DNP IgM titer in the pre-inoculation plasma sample and in all subsequent samples. The occurrence of natural antibodies to DNP and weak DNP binding by non-immunoglobulin plasma proteins has been reported previously in the green turtle (Benedict & Pollard, 1972). IgM titers are notoriously difficult to measure in an ELISA format (Betts Carpenter, 1992; Kuen et al., 1993). Problems can include antibody aggregate formation, nonspecific binding of IgM to ELISA

plates, and autoimmune anti-Ig IgM (RF factors). Nonspecific IgM binding was reduced by using high salt buffers (1% BSA in PBS adjusted to 0.5 M NaCl) and using Polysorp ELISA plates. Despite these efforts, the anti-DNP IgM activity of the pre-inoculation sample remained high. The 2 turtles immunized repeatedly with 200 µg doses of DNP-BSA did not develop detectable anti-BSA titers in this study. The results may be explained by the antigen dose that was used, because a previous study (Benedict & Pollard, 1972) that demonstrated anti-BSA titer development used pure BSA at much higher doses (18-80 mg).

The competitive inhibition experiments were designed to demonstrate that the putative anti-DNP responses detected in the immunization experiment were due to increasing plasma levels of anti-DNP specific antibodies. Incubation of high anti-DNP titer plasma with increasing concentrations of soluble DNP antigen prior to incubating the plasma with bound antigen inhibited the ELISA reaction and clearly demonstrated that the turtle antibody responses being measured were predominantly and specifically directed against the DNP hapten on the DNP-BSA antigen. Similarly, increasing concentrations of rabbit anti-DNP specific antibodies competitively inhibited the ELISA reactions of high anti-DNP titer turtle plasma.

A surprising result of this study was the isolation of a monoclonal antibody (HL814) with specificity for 5.7S IgY

heavy chain. Previous work with 5.7S and 7S immunoglobulins of turtles using polyclonal antisera revealed a close antigenic relationship between these two classes (Benedict & Pollard, 1972). The 5.7S heavy chain is believed to be a fragment of the 7S molecule. Molecular analysis of the sequences of 5.7S and 7S heavy chains of ducks, Anas platyrhynchos indicate that, except for the extreme carboxyl terminus of the 5.7S molecule, which is unique, the 5.7S constant region sequences (CH1 and CH2) are virtually identical to its 7S counterpart (Magor et al., 1992). The duck 5.7S molecule is a truncated version of the 7S molecule (lacking 2 terminal exons), most likely derived by differential mRNA splicing. If the relationship between green turtle 5.7S and 7S heavy chains is the same as for the duck, then it should be possible to develop Mabs that recognize epitopes that are common to both 5.7S and 7S and Mabs that are specific for epitopes unique to 7S. Mabs specific for a unique 5.7S epitope should be relatively rare and should recognize the unique terminal portion of the molecule. The data support the fact that Mab HL814 is 5.7S specific. Mab HL814 detects increasing antigen-specific antibody titers in immunized turtles and binds to a 35 kD protein that forms a 120 kD native structure possessing an immunoglobulin light chain. A partial amino acid sequence for this protein is needed to begin studying the homologies of this protein. Mab HL814 is a key reagent for affinity purification of green

turtle 5.7S immunoglobulin and the subsequent cloning and sequencing of the 5.7S cDNA for comparative studies with other vertebrate immunoglobulins.

The primary objective for developing these Mabs was to design practical serodiagnostic tests for monitoring populations of endangered green turtles for exposure to pathogens and for studying humoral immune competence in this species. However, the time and effort required to develop and validate these monoclonal antibody reagents warranted a preliminary assessment of their applicability for other threatened/endangered sea turtle species. Preliminary screening indicated that while the IgM and 5.7S specific Mabs were species specific, the 7S IgY specific and light chain Mabs cross reacted with other sea turtle species. Thus, these Mabs may be used to explore humoral immune function and conduct seroepizootiologic studies in all of these species.

CHAPTER 4
EXPERIMENTAL TRANSMISSION OF GREEN TURTLE FIBROPAPILLOMATOSIS

Introduction

The etiology of green turtle fibropapillomatosis (GTFP) is unknown and possible causes include one or more infectious and non-infectious agents (Chapter 2). However, the pattern of disease spread during GTFP outbreaks among captive green turtles (Hoffman & Wells, 1991; Jacobson, 1981b; Jacobson et al., 1989) is consistent with an infectious etiology; therefore, experiments to test this hypothesis and to distinguish among potential infectious agents were undertaken. Successful experimental transmission of GTFP from affected to unexposed individuals would prove that a transmissible (infectious) agent is responsible for the disease. Furthermore, experimental induction of GTFP in previously unexposed individuals with a purified candidate agent found in donor tumors would prove that the agent causes this disease, fulfilling Koch's postulates.

This chapter presents the findings of a series of controlled transmission experiments, conducted over 3 years, designed to test the hypothesis that GTFP is caused by a transmissible agent. The work focused on two types of agents: either 1) spirorchid ova as proposed by Harshbarger (1984),

or 2) a subcellular (filterable) infectious agent, such as a virus.

Materials and Methods

Three separate transmission studies were started between December 1991 and September 1993. Each successive study was designed to test a new or modified hypothesis, using information learned from the previous study, and to optimize use of the limited supply of experimental animals. Thus, specific materials and methods for each study are presented separately below.

Animal Housing and Maintenance

All transmission experiments were conducted at a sea turtle rehabilitation facility in the Florida keys (The Turtle Hospital, Marathon, FL). This facility had a large artificial pond (converted from a salt water swimming pool) that was used to house injured sea turtles and green turtles with GTFP. Water in this pond exchanged freely with adjacent Florida Bay by tidal action. In addition, several large fiberglass tanks (useable capacity, 2400 l) were available for housing experimental turtles in isolation. These tanks were on a continuous flow system that provided approximately 20 volume changes per day of sand filtered (TA60 sand filters, PACFAB, Longwood, FL, USA) Florida Bay water. The filters were back flushed daily. Tanks were vacuumed weekly and thoroughly cleaned monthly. Water temperatures, pH, and

salinity (specific gravity) were monitored and recorded weekly.

All experimental turtles were fed floating trout chow (Ralston Purina, St. Louis, MO, USA). The amount fed ranged from 1.5 to 1.75% body weight per day and was adjusted to provide for moderate growth, prevent obesity, and minimize cannibalism.

Experimental turtles were individually identified by a system of notches cut in one or more marginal scutes. In all studies, turtles were monitored for over 12 months following any experimental treatment. Turtles were visually inspected daily and palpated weekly for development of inflammation or masses and observations were recorded weekly. Blood samples for minimum health assessment (packed cell volume) and plasma banking were collected from each turtle prior to any experimental treatments and periodically (every few months) thereafter throughout the monitoring period. Approximately 1-3 ml of blood was collected from the dorsal cervical sinus using manual restraint (Owens & Ruiz, 1980).

First Transmission Study (1991)

Materials for inoculation (1991)

Spirorchid trematode ova. Intact spirorchid trematode ova were selected by hand from macerated spleen and liver tissue of green turtles that were presented for necropsy. Ova from two species were identified and collected, fusiform ova conforming to Learedius learedii and ellipsoid ova of an

unidentified species, possibly Neospiroorchis sp. (Ellis Greiner, University of Florida, Gainesville, FL 32610, pers. comm.; see Chapter 7). Aliquots of 50 ova were suspended in 100 μ l of sterile saline and kept refrigerated until used.

Tumor preparations. Three fibropapillomas were surgically removed using local anesthesia from a single free-ranging immature green turtle (CM92-14) that had stranded in the Florida keys. Tumors were scrubbed with a sterile brush and copious amounts of sterile saline solution. Small pieces of intact tumor (approximately 1 g wet weight) were surgically implanted under the skin of recipient turtles. Small (1 g) dermal plugs, tumors that were trimmed of epidermis, were also implanted. The remaining fibropapilloma pieces (approximately 5 g) were ground in a Ten Broeck glass tissue grinder in approximately 10 ml ice cold saline. This preparation was drawn up through successively smaller hypodermic needles (down to 25 gauge) to eliminate large debris. A portion of this crude homogenate was used for inoculation. The remaining homogenate was frozen on dry ice and held frozen for 15 minutes before being thawed and refrozen. This material was thawed and ground a second time prior to inoculation.

Experimental turtles (1991)

Nine green turtles were raised in captivity at Sea World, Orlando Florida from 12 hatchlings provided by the Florida Department of Environmental Protection. At 6 months

of age these turtles were transported to Marathon, Florida where they were housed in isolation in one of the fiberglass tanks until experiments were begun. The turtles were approximately 1 year old and ranged in size from 20 to 25 cm straight carapace length when experiments were begun.

Experimental treatments (1991)

Three turtles were used to determine whether GTFP could be readily transmitted to unexposed individuals via water or direct contact with affected turtles. These turtles were housed in a wire mesh pen within the artificial pond that was completely open to unfiltered Florida Bay water and which housed several turtles with spontaneous GTFP. Limited physical contact between experimental turtles and turtles with GTFP was possible. The remaining experimental turtles were housed in 3 separate fiberglass tanks (up to 3 turtles per tank). In one tank all 3 turtles were injected intradermally (i.d.) and subcutaneously (s.c.) at each of 3 sites, using a 0.5 ml insulin syringe with a 27 gauge needle, with aliquots of 50 intact spirorchid trematode ova suspended in 100 μ l saline. Inoculation sites included the upper eyelid, proximal margin of a large scale on the palmar surface of the front flipper, and proximal margin of a large scale on the dorsal surface of the rear flipper. Sites on the left side of the body were injected with Learedius learedii ova whereas comparable sites on the right side were injected with Neospirorchis-like ova. Two of these turtles received

booster injections of each type of ova a month later. A second tank housed 2 turtles that were treated with each of the various GTFP preparations, including intact tumor implants (s.c. into right and left rear limbs), dermal plugs (s.c. into right and left forelimbs), unfiltered homogenate (100 μ l i.d. and s.c. into the left eyelid and into scarified neck skin), and twice frozen and thawed homogenate (100 μ l i.d. and s.c. into the right eyelid). Skin scarification was performed with an 18 gauge needle and the inoculum was worked into the tissue by multiple needle pricks with a 25 gauge needle and allowed to air dry for at least 15 minutes before turtles were returned to water. The remaining turtle received only sterile saline injections and was isolated in a third tank to serve as a control for the water filtration system.

Second Transmission Study (1992)

The purpose of the second study was to passage one of the experimentally induced tumors in vivo and determine if it contained a transmissible agent that could pass through a 0.2 μ m filter, thus ruling out the possibility that tumor induction occurred by transplantation of viable neoplastic cells.

Material for inoculation (1992)

The experimentally induced palpebral tumor from one turtle (TX91-GT#5) was removed surgically and a 4 g sample was ground in 10 ml ice cold saline and twice frozen and thawed, as described above, to produce a crude unfiltered

homogenate. A portion of this material was used for inoculation. The remaining portion was filtered successively through three 0.2 μm syringe tip filters (Acrodisc®, Gelman Sciences, Ann Arbor, MI) before being inoculated into turtles.

Experimental turtles and treatments (1992)

The only turtles available for experimentation were the control and 6 transmission negative turtles from the previous study. Two turtles, one that previously had been injected with spirorchid ova and one that had been contact exposed, were injected with unfiltered homogenate (100 μl s.c. into right eyelid, forelimb, and rear limb). Three turtles, one previously injected with ova, one contact exposed, and the control, were inoculated with the (0.2 μm) filtered homogenate (100 μl s.c. into right eyelid, forelimb, and rear limb).

Third Transmission Study (1993)

This study was designed to determine if GTFP could be transmitted as a cell-free (0.45 μm) filterable extract and to gather some preliminary data on the sources of variation in transmissibility.

Material for inoculation (1993)

Donor turtles. Four free-ranging juvenile green turtles (35-59 cm SCL) with GTFP that stranded or were collected in Florida Bay or the lower Florida Keys were used as tumor donors in the 1993 study (Table 4-1). GTFP was severe enough

Table 4-1. Free-ranging green turtles with cutaneous fibropapillomatosis used as fibropapilloma donors

Identity	Recovery Date ^a	Location (Florida, USA)	SCL (cm)	Wet Weight of Tumor Pool (g)
Donor #1 (Flamingo)	28 Jun 93	Monroe Co., Florida Bay	35.6	9.0
Donor #2 (Everglades)	30 Jun 93	Monroe Co., Florida Bay	61.4	19.0
Donor #3 (Pappy)	25 Aug 93	Monroe Co., Stock Island	58.5	26.3
Donor #4 (Coastie)	21 Aug 93	Monroe Co., Bahia Honda Bridge	59.3	15.4

^a Date of capture or stranding.

in these turtles to justify their removal from the wild for rehabilitation. Each had multiple cutaneous fibropapillomas ranging in size from a few millimeters to over 9 cm in diameter involving the axillary and inguinal regions, front and rear flippers, neck, and eyelids. Two turtles, donor #2 (Everglades) and donor #4 (Coastie) were unable to maintain neutral buoyancy and floated high in the water. Both subsequently died and were found on necropsy to have multiple pale firm nodules (fibromas) in visceral organs including lungs, kidney, liver, and heart. The other two donor turtles were surgically treated and subsequently released.

Tumor collection. General anesthesia was induced in each donor turtle with a mixture of isoflurane (Aerrane®, Anaquest, Madison, WI, USA) and nitrous oxide in oxygen delivered by mask and then by endotracheal tube. Once anesthetized, the turtles were draped and tumors were scrubbed with a sterile brush and copious amounts of sterile saline. All cutaneous tumors were excised and incisions were closed with 2.0 nylon suture material (Dermalon®, American Cyanamid, Danbury, CT, USA). Highly arborizing fibropapillomas with intact epithelium were selected for use in the transmission experiments. Ulcerated and necrotic masses were discarded. Each tumor was cut into quarters. Representative sections were taken from each quarter and either fixed in 10% buffered formalin or immersed in OCT medium (Tissue-Tek®, Miles Inc., Elkhart, IN, USA) and frozen

in liquid nitrogen. A portion of each mass was retained for tissue culture studies. The remaining pieces were placed in a sterile cup, weighed, and stored on dry ice until processing.

Preparation of cell-free tumor homogenates. Tumor homogenates were prepared from pooled tumor pieces (9.0 to 26.3 g total wet weight) taken from each donor (Table 4-1). The pooled tumor fragments from each donor were thawed, minced with a sterile scalpel and scissors, then ground in a blender (Osterizer®, Sunbeam-Oster, Schaumburg, IL, USA) in chilled sterile saline. This material was further homogenized in Ten Broeck glass tissue homogenizers (Pyrex, Corning, NY, USA) on ice. The crude homogenate was frozen on dry ice and thawed before being homogenized a second time in the blender. The freeze-thaw cycle was then repeated. Sufficient sterile saline was added during homogenization to produce a final 33% w/v crude homogenate.

The twice frozen and thawed crude homogenate was centrifuged in a clinical centrifuge at 500 x g for 10 minutes to sediment large debris and the supernatant was collected. Centrifugation of the pellet was repeated once and the supernatant added to that previously collected. The supernatant was then centrifuged at 10,000 x g for 10 to 15 minutes in a Savant microcentrifuge to pellet cells and debris. Samples of this supernatant were examined microscopically to confirm the absence of intact cells.

Approximately half of the cell-free supernatant was filtered through a 0.45 μm syringe tip filter (Acrodisc®, Gelman Sciences, Ann Arbor, MI, USA) to remove contaminating bacteria and any remaining intact cells. The rest of the cell-free supernatant was used as an unfiltered extract. The final centrifugation pellets and aliquots of unfiltered and filtered supernatants were frozen in liquid nitrogen and stored at -150°C in a liquid nitrogen freezer for future analysis.

Experimental turtles (1993)

Twenty captive-reared green turtles were used in this study. Five to seven eggs were collected from each of 4 green turtle nests that were deposited by different females within a one week period in August 1992 on Melbourne Beach, Brevard County, Florida. Three of the four nesting females were identified by flipper tags (Steven Johnson, University of Central Florida, Orlando, FL 32816, pers. comm.). Following oviposition, each nest was marked and eggs were left in place until they were within 1-10 days of their expected hatching date (Rebel, 1974). Eggs were then transported in separate plastic boxes filled with beach sand to a laboratory incubator (28.5°C and 95-100% relative humidity) where they remained through hatching, which occurred approximately 52 days post-oviposition (range 49-57 days). Hatchlings were individually marked and housed by clutch in plastic tubs filled with filtered sea water. Hatchlings were fed a

commercial pelleted diet (Reptomin®, TetraWerke, Melle, Germany) for approximately 2 weeks. Then the turtles were transported by air to Marathon, Florida where they were housed by clutch in 4 separate fiberglass tanks. Twenty turtles (5 from each clutch) were raised until they were between 9 and 11 months old before experiments were begun.

Experimental treatments (1993)

Four replicate transmission experiments were conducted. In each experiment, tumor extract from a single donor was used to treat one recipient from each of the 4 clutches. Treatments included intradermal injections into the upper eyelid (100 μ l), palmar surface of the front flipper (200 μ l), dorsal surface of the rear flipper (200 μ l), and instillation of 100 μ l into scarified skin of the neck and shoulder. All treated turtles received inoculations of filtered tumor extract on the right side of the body. In addition, half of these turtles received sterile saline (sham) inoculations and half received unfiltered extract inoculations at comparable sites on the left side of the body. On the neck, turtles received either unfiltered or filtered tumor extract. One turtle from each clutch was maintained as a control (sentinel). Sentinel turtles received no inoculations but were housed with their treated clutch-mates to control for spontaneous disease or waterborne and contact transmission by the putative infectious agent.

Histopathology

Biopsies of normal skin and putative experimentally induced tumors were collected, using a 6 mm biopsy punch or scalpel blade under 2% lidocaine local anesthesia, and fixed in neutral buffered 10% formalin for histopathologic examination. Tissue specimens were processed routinely through graded alcohols and xylene and embedded in paraffin. Standard 6 μm sections were made and stained with hematoxylin and eosin (H&E).

Transmission Electron Microscopy

Specimens for electron microscopy were punched from formalin-fixed paraffin-embedded fibropapillomas, post-fixed in osmium tetroxide, and embedded in Spurr's resin. Ultrathin sections were placed on copper grids and stained with uranyl acetate and lead citrate and examined on an electron microscope.

Negative Staining Electron Microscopy

Samples of 0.45 μm filtered tumor extracts that had successfully transmitted GTFP in the 1993 study were examined for the presence of virus-like particles as follows. First, 10 μl samples of filtered tumor extract were applied to carbon coated 400 mesh copper grids and allowed to adsorb for 10 to 30 seconds. The grids were drained of excess liquid with a filter paper wick and then immediately floated on a drop of 2% aqueous uranyl acetate for 30 seconds. The grids were drained of excess fixative and allowed to air dry before

examination in the electron microscope. Second, 1 ml of filtered tumor extract was centrifuged at 12,000 x g for 20 minutes. The clarified supernatant was then centrifuged at 100,000 x g for 2 hrs in an Airfuge A-100/18 rotor (Beckman Instruments, Fullerton, CA, USA). The pellet was resuspended in 40 μ l distilled water and aliquots were adsorbed to carbon coated 400 mesh copper grids and prepared for electron microscopy as described above.

Results

First Transmission Study (1991)

Fibropapillomas did not develop in any of the 3 water and contact exposed turtles housed in the artificial pond or in the control turtle housed in isolation after more than 9 months of monitoring. Tumors did not develop in any of the 3 turtles treated with spirorchid ova. In contrast, both tumor-treated turtles developed single tumors on the right upper eyelid where they had been injected with 100 μ l of twice frozen and thawed tumor homogenate. Tumors did not develop anywhere else on these 2 turtles. Although small skin swellings were noted at some sites as early as 4 weeks post-inoculation, these did not progress and were interpreted as inoculation scars. The eyelid tumors first became apparent as small (1-2 mm) raised swellings approximately 5-6 months after inoculation. These tumors grew rapidly during the summer months and had to be surgically debrided multiple times. Eighteen months post inoculation, after multiple

attempts to obliterate the experimentally induced eyelid tumor, one turtle (TX91-#4) developed cutaneous tumors at previously uninoculated sites on the plastron, tail, neck, and front flipper. Both turtles eventually died from anesthetic or post-surgical complications approximately 15 and 21 months post inoculation.

Second Transmission Study (1992)

The three turtles that had been kept as controls for the 1992 study never developed tumors during the subsequent 15 months of monitoring. None of the three turtles inoculated with 0.2 μ m filtered homogenate, derived from the experimentally induced tumor, developed tumors during the same time period. However, 2 of the 3 turtles treated with unfiltered homogenate developed tumors at 1 of 3 injection sites, the right rear limb. These tumors developed subcutaneously and had grown to > 1 cm in diameter before they became clinically apparent, approximately 1 year after inoculation, as raised masses. These masses were removed surgically and the turtles recovered without further problems.

Third Transmission Study (1993)

Four independent transmission experiments were conducted on the following dates: 6-7 July 1993 (groups #1 and #2 using homogenates from donors #1 and #2 respectively), and 3-4 September 1993 (groups #3 and #4 using homogenates from donors #3 and #4 respectively). Table 4-2 shows the results

Table 4-2. Fibropapilloma development at inoculation sites in green turtles treated with filtered cell-free fibropapilloma extracts

Clutch	Experiment #1 (Donor #1)	Experiment #2 (Donor #2)	Experiment #3 (Donor #3)	Experiment #4 (Donor #4)	Control Group
A	+	(2/4) ^a	+	(3/5)	-
B	+	(2/4)	+	(4/4)	N/A ^b
C	+	(1/5)	+	(2/4)	-
D	+	(3/5)	+	(3/4)	-

^a "+" indicates tumor growth at one or more inoculation sites and "-" indicates no tumor growth at any treatment site. Data in parentheses are the number of anatomic sites where tumors developed over the number that were inoculated with 0.45 µm filtered cell-free tumor extracts. Sixteen turtles were inoculated by injection or scarification at 4 or 5 sites on the right side of the body with filtered extracts prepared from 4 donor turtles with spontaneous tumors. The control group was not inoculated.

^b N/A, this control turtle died before experiments were begun.

of inoculations with filtered tumor extracts after 10 and 12 months respectively. One control (sentinel) turtle died from a bite wound infection before the 1993 experiments were begun. The three surviving control turtles did not develop spontaneous tumors during this period. All twelve turtles inoculated with tumor extracts from donors 1, 2, and 3 developed tumors at one or more injection sites. The 4 turtles in group #4, that received tumor extract from donor #4, did not develop tumors. Tumors developed only at sites that were inoculated with GTFP extracts (Figure 4-1). Tumors did not develop at sham inoculated sites or at any uninoculated sites. Although several turtles incurred bite wounds on rear flippers and tails, fibropapillomas did not develop at these locations.

Table 4-3 lists the frequencies of fibropapilloma development by the type of inoculum and route of inoculation. Filtered GTFP extracts were no more successful than unfiltered extracts in inducing tumor development (58% of injection sites and 17% of scarification sites in 16 turtles versus 42% of injection sites and 19% scarification sites in 8 treated turtles). The differences in percent success between filtered and unfiltered extracts were not statistically significant (Chi-Square = 1.46, $p > 0.10$). On the other hand, sites injected intradermally were more likely to develop tumors than scarification sites (Chi-Square = 13.4, $p < 0.001$).



Figure 4-1. Experimentally induced cutaneous fibropapillomas in green turtles. Tumors developed at one or more inoculation sites in 12 recipient turtles from 3 of 4 independent 1993 transmission experiments. Representative tumors (arrows) illustrate site specific development and gross appearance at the time of biopsy. Recipient TX93-C3 (upper left) with sessile, verrucous tumor on right upper eyelid induced by intradermal injection of filtered tumor extract. Recipient TX93-C2 (upper right) sessile, smooth tumor on right front flipper induced by intradermal injection of filtered tumor extract. Recipient TX93-B3 (lower left) sessile, verrucous tumor on right shoulder induced by scarification with filtered tumor extract. Recipient TX93-C3 (lower right) pedunculated, verrucous tumor on right rear flipper induced by intradermal injection of filtered tumor extract.

Table 4-3. Frequency of fibropapilloma development at injection and scarification sites in recipient green turtles

Inoculum	Injection Sites ^a	Scarification Sites ^b
Unfiltered Tumor Extracts	10/24 (42%) ^c	3/16 (19%)
Filtered Tumor Extracts	28/48 (58%)	4/24 (17%)

^a Data are number of sites that developed tumors over the total number of sites receiving each treatment. Intradermal injection of 0.1-0.2 ml of inoculum at 3 sites (upper eyelid, front, and rear flippers) on each turtle.

^b Application of 0.1 ml of inoculum to scarified skin on neck and shoulders.

^c Data include turtles from experiment #4 in which tumors did not develop at any inoculation sites.

Time to Tumor Development

The two tumor positive turtles in the 1991 transmission study developed clinically apparent tumors approximately 5-6 months post-inoculation following an increase in water temperatures from below 24°C to above 28°C. The best data, however, for describing the time to tumor development comes from the 1993 study.

Figure 4-2 shows the time course of tumor development for individual turtles during the 1993 study. Earliest indications of tumor development were identified as slightly raised epidermal swellings ranging from 0.5 to 8 mm maximum diameter. The earliest tumors were detected at 15 weeks post inoculation in two turtles from replicate #3. The time lag between inoculation and observation of the first tumors to develop on individual turtles ranged from 14.6 to 43.4 weeks ($\bar{x} = 26 \pm 8.8$ weeks). The average lag time for first tumor detection was 8 weeks longer for the 8 turtles in the first set of replicates (July) ($\bar{x} = 28.6 \pm 8.4$) than for the 4 turtles in the second set (September) ($\bar{x} = 20.6 \pm 7.7$ weeks), although this difference was not statistically significant (Mann-Whitney U test, 2-tailed, $0.05 < p < 0.10$). The average mean tumor detection time (averaged for all tumors on each individual) was 6.8 weeks longer for the 8 turtles in the first set ($\bar{x} = 33.4 \pm 5.2$) than for those in the second set ($\bar{x} = 26.6 \pm 4.3$ weeks) and this difference was statistically significant (Mann-Whitney U test, 2-tailed, $p < 0.05$). This

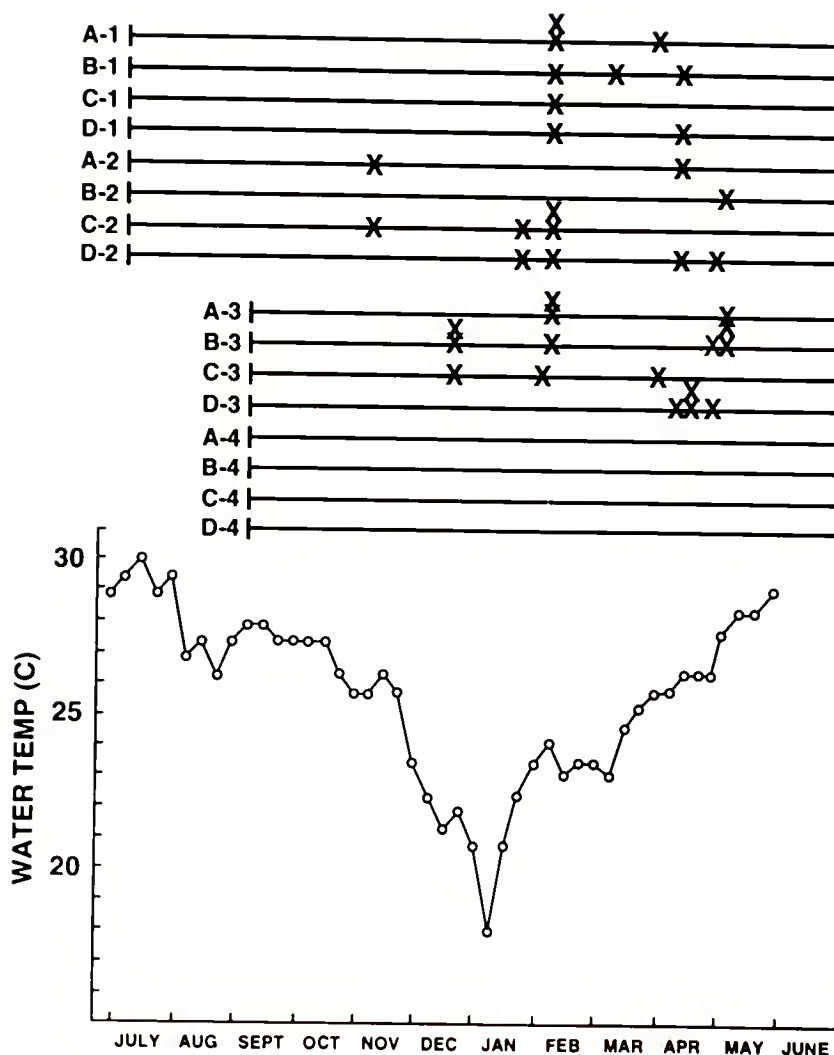


Figure 4-2. Time course for experimental fibropapilloma induction during the 1993 transmission study. Horizontal parallel lines above the XY graph represent the observation periods for each recipient turtle (TX93-A1 to TX93-D4) beginning with the dates of their inoculation with tumor homogenate. Dates on which tumors were first detected are indicated by X's. The XY graph indicates the fluctuation in water temperature (recorded weekly) during the course of this experiment.

difference roughly corresponds to the 8 week gap between sets of replicate experiments and suggests that tumors tended to appear synchronously. The majority of fibropapillomas (29 out of 36) were initially detected between early February and May regardless of when turtles were inoculated. Only 4 turtles had tumors develop within the first 4 months post inoculation. During the course of the experiment water temperatures ranged from 30°C in the summer to 17.5°C in the winter and were lowest (below 21°C) from December through January (Figure 4-2). Salinity and pH remained constant (1.027 sp. gr. and 8.2 respectively). The onset of colder water temperatures in December appeared to retard growth rates of early tumors. A similar effect on subclinical tumors could synchronize their appearance with the return to warmer water temperatures.

Histopathology of Experimentally Induced Tumors

The 4 experimentally-induced tumors from the first 2 studies were confirmed histologically to be fibropapillomas and fibromas. In the 1993 study, 38 experimentally induced fibropapillomas from 11 recipient turtles were biopsied between 1 and 28 weeks following their detection. Lesions were raised, sessile or polypoid masses with verrucous or smooth surfaces and ranged from 0.5 to 2 cm in diameter when biopsied (see Figure 4-1). Tumors consisted of epidermal hyperplasia supported by arborizing proliferating fibrovascular stroma (Figure 4-3). The histologic features of

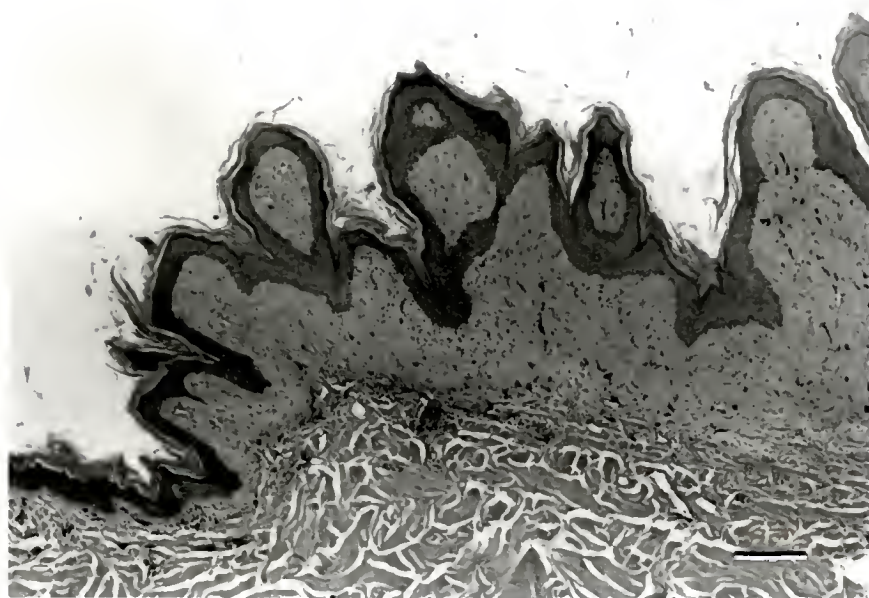


Figure 4-3. Experimentally induced fibropapilloma showing characteristic benign epidermal hyperplasia on broad fibrovascular stalks. Experimental tumors did not contain trematode eggs. H&E (scale bar = 200 μ m).

these induced lesions were consistent with spontaneous green turtle fibropapillomas (Jacobson et al., 1989; see Chapter 2) and are described more fully in chapter 6. No trematode ova were observed within any sections.

Scattered foci of ballooning degeneration were observed within the spinous layer of the epidermis in several biopsy sections the 1993 study (Figure 4-4 A). Degenerating keratinocytes were hypertrophic and vacuolated. Eosinophilic intranuclear inclusions (Figure 4-4 B,C) were identified within some foci of ballooning degeneration in 5 biopsies from 4 turtles. In other foci of epidermal degeneration, most cells had pyknotic nuclei.

Transmission Electron Microscopy

Intranuclear inclusions within degenerating keratinocytes contained virus-like particles ranging from 80 to 90 nm in diameter. These intranuclear particles were in various stages of assembly ranging from empty capsids to intact nucleocapsids with electron dense cores (Figure 4-5 A). These immature particles were observed in the process of budding through the nuclear membrane (Figure 4-5 B). Mature enveloped particles in the cytoplasm measured 110 to 125 nm (Figure 4-5 C).

Negative Staining Electron Microscopy

No morphologically distinct virus-like particles could be demonstrated in samples of transmission positive tumor extracts or in ultracentrifuge pellets prepared from 1 ml

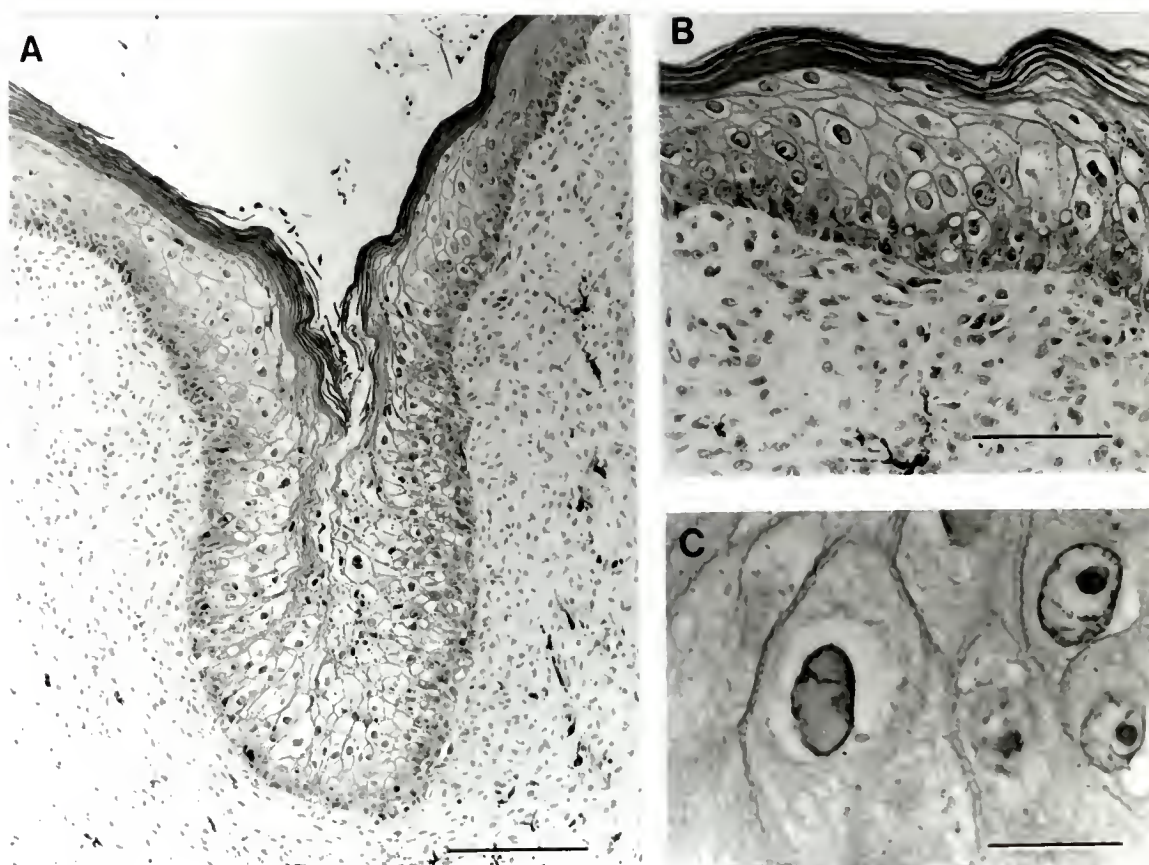


Figure 4-4. Cytopathology in the epidermis of experimentally induced green turtle fibropapillomas. (A) Focal ballooning degeneration in the epidermis. H&E (scale bar = 200 μm). (B) Higher magnification showing intranuclear inclusions within degenerating keratinocytes. H&E (scale bar = 100 μm). (C) Oil immersion showing intranuclear inclusion and perinuclear cytoplasmic clearing. H&E (scale bar = 20 μm).

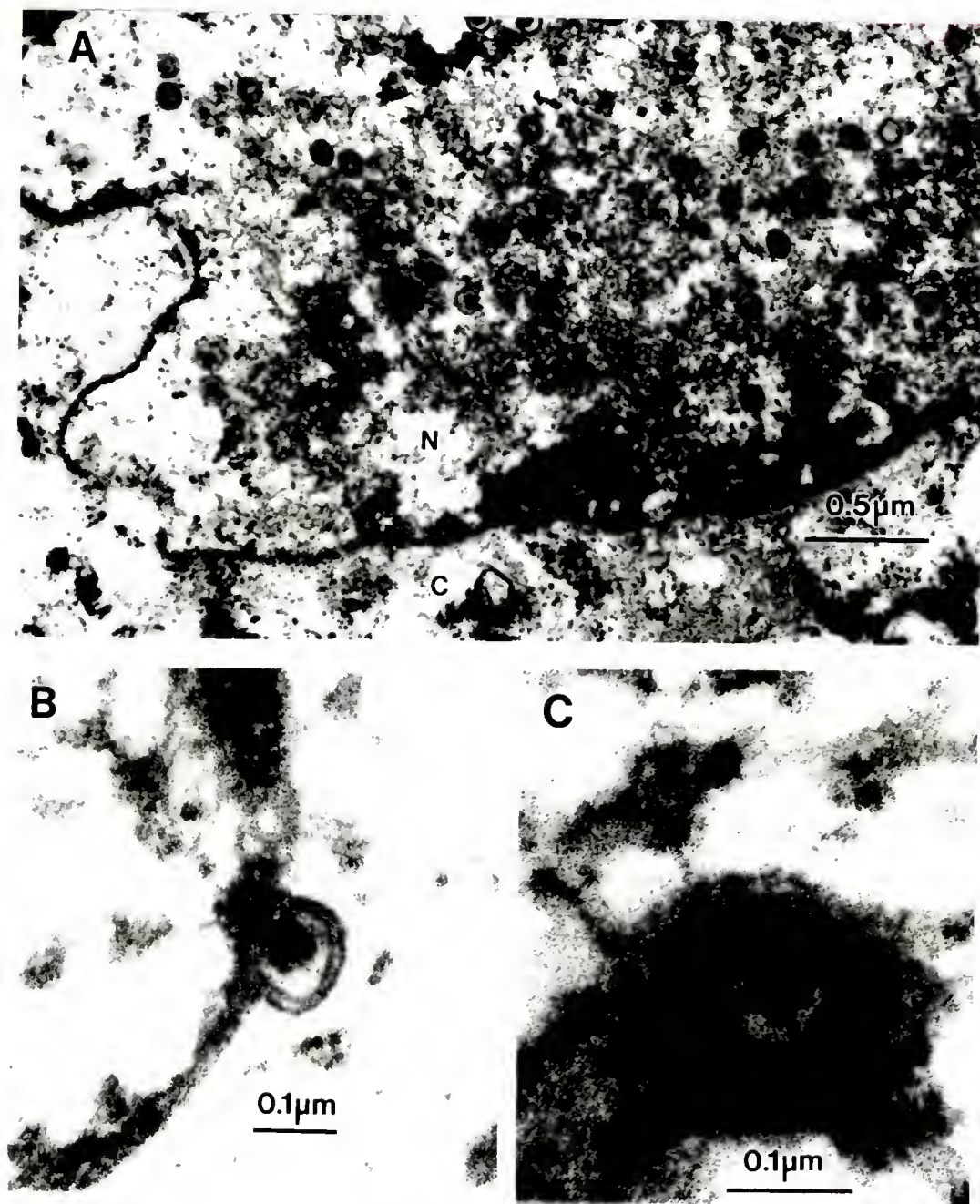


Figure 4-5. Herpesvirus-like particles in experimentally induced fibropapillomas. (A) Herpesvirus-like particles in various stages of development within the nucleus (N = nucleus, C = cytoplasm). Both empty capsids and complete nucleocapsids containing electron dense cores can be seen. (X 37,000). (B) Virion budding through the nuclear membrane. (X 128,000). (C) Mature enveloped virion within the cytoplasm. (X 195,000).

aliquots of these extracts by negative staining electron microscopy.

Discussion

Experimental Evidence for an Infectious Etiology

The first transmission study conducted in 1991 demonstrated that GTFP was transmissible as a twice frozen and thawed homogenate. Although the freeze-thaw cycles should have lysed cells, passage of intact and viable tumor cells could not be ruled out. Thus, there were two alternative explanations for these data: 1) successful transplantation of neoplastic cells similar to canine transmissible venereal tumor (Nielsen & Kennedy, 1990) or 2) transmission of an infectious agent. Because the inocula were not filtered, the putative infectious agent could range in size from microscopic (virus, bacteria, fungi) to macroscopic (spirorchid ova).

The second transmission study tested whether the putative transmissible agent was able to pass through a filter designed to exclude particles $> 0.2 \mu\text{m}$. The results indicated that although an experimentally induced tumor could be transmitted as a twice frozen and thawed unfiltered homogenate, the agent responsible for transmission was retained by the $0.2 \mu\text{m}$ filters. Alternate explanations for these results are: 1) the agent was larger than $0.2 \mu\text{m}$ effective diameter (a large virus or intact cell), 2) the agent was $< 0.2 \mu\text{m}$ but was tightly associated with cellular

debris, or 3) nonspecific binding (charge effects) of the agent to the filter matrix was sufficient to retain nearly all of the agent after 3 successive filtrations.

Failure of the earlier studies to resolve whether GTFP was transmissible as a cell-free filtrate prompted the third transmission study. In this study fibropapillomas were induced in twelve healthy captive-reared juvenile green turtles using both unfiltered and 0.45 μm filtered cell-free extracts prepared from fibropapillomas collected from 3 out of 4 donors with spontaneous disease. These results provide the first experimental evidence that GTFP is caused by an infectious agent under 0.45 μm in size. Because the filtration step eliminated fungi and most bacteria (except Mycoplasma spp.) the infectious agent is most likely a virus.

Role of Spirorchid Ova

The hypothesis that GTFP is caused by spirorchid ova (Harshbarger, 1984) was not supported by any of the experimental evidence. In the first study, tumors did not develop in 3 turtles that were inoculated with ova of 2 spirorchid species commonly found in tumors, although it is possible that the dosage of eggs and the dosing frequency (up to 2 inoculations) may have been insufficient to elicit a response. In the second transmission study, tumors were induced with a tumor extract that could not contain intact ova or soluble egg products because the donor was proven by necropsy, examination of tumor digests, and ELISA to be

spirorchid negative (see Chapter 7). Finally, the centrifugation and filtration steps used in the third transmission study ruled out the possibility that spirorchid ova were involved in the induction of fibropapillomas.

The GTFP-Associated Herpesvirus

The experimentally induced tumors were morphologically identical to published descriptions of naturally occurring GTFP (see Chapter 2). More detailed comparison with spontaneous tumor histopathology will be presented in a subsequent chapter (Chapter 6). The herpesvirus-like particles, found in eosinophilic intranuclear inclusions within scattered foci of epidermal ballooning degeneration, were similar in size and morphology to those described by Jacobson et al. (1991) from 2 green turtles with GTFP. Except for those 2 spontaneous cases (Jacobson et al., 1991), this study provides the only direct evidence that a specific virus may cause GTFP.

The significance of the association between this herpesvirus and spontaneous or experimental GTFP remains unclear. Herpesviruses have been found in cutaneous tumors in a variety of species (see Chapter 2). Two other herpesviruses have been reported in green turtles, including the virus that causes grey patch disease (Rebell et al., 1975) and a herpesvirus associated with respiratory disease (Jacobson et al., 1986a). Because herpesviruses have a tendency to colonize or recrudesce in tumors and tissues of debilitated

animals it is possible that their presence in fibropapillomas represents a secondary infection, unrelated to the primary disease process, and possibly co-transmitted with the true GTFP agent. On the other hand, herpesviruses have been shown to cause neoplasia in several species. Examples include Oncorhynchus masou virus in salmonids and Cyprinid herpesvirus in carp (Anders & Yoshimizu, 1994), Ranid herpesvirus (Lucké renal adenocarcinoma) in leopard frogs (McKinnell, 1981, 1984), Gallid herpesvirus 1 (Marek's disease) in chickens (Powell, 1985), Herpesvirus saimiri 2 (lymphoma) in new world primates (Trimble & Desrosiers, 1991), and Epstein-Barr virus (Burkitt's lymphoma) in humans (Henle & Henle, 1985). We cannot conclude that this herpesvirus is the etiologic agent of GTFP until it has been isolated and proven to be oncogenic in transmission experiments. Meanwhile other virus types that have been associated with or shown to cause proliferative skin lesions in vertebrates must remain on the list of potential etiologic agents for GTFP (see Chapter 2).

Attempts to Identify Viruses in Donor Material

Preliminary attempts to identify virus particles in samples of transmission positive cell-free tumor extracts using negative staining electron microscopy were unsuccessful. In theory this would have allowed morphologic descriptions of all viruses in the extract. One explanation is that the agent was destroyed during sample preparation.

Enveloped viruses such as herpesviruses and retroviruses are sensitive to storage and processing conditions and, although crude extracts were stored at -150°C , no attempt was made during processing to protect the agent from proteolytic enzymes. Another possibility is that the concentration of intact viral particles within the sample was below the detection limits of the method. Successful detection by negative staining electron microscopy requires approximately 10^6 to 10^9 virus particles per ml (Doane, 1992). This was probably the case because the herpesvirus described above was found in scattered foci within a very small percentage of epidermal cells. Similarly, papillomavirus vegetative replication often occurs only sporadically in the most superficial terminally differentiated epidermal cells of a papilloma (Howley, 1990). A third possibility is that the agent that causes GTFP is present in tumors primarily as episomal genetic material that is infectious under experimental conditions, as shown for several papovaviruses, including hamster polyomavirus (Graffi et al., 1969), cottontail rabbit papillomavirus (Brandsma & Xiao, 1993; Ito & Evans, 1961), and some herpesviruses (Roizman & Furlong, 1985). Several oncogenic viruses, including papillomaviruses (Howley, 1983; 1990) and retroviruses (Benjamin & Vogt, 1990) can cause tumors in tissues that are not permissive to vegetative viral replication. Equine sarcoids, for example, are fibromatous tumors that are believed to be caused by

bovine papilloma virus infection, but while intact or partial viral genomes are found within sarcoid fibroblasts, virion production never occurs (Amtmann et al., 1980; Angelos et al., 1991; Lancaster et al., 1977). A fourth but less probable explanation for the inability to detect virus particles in tumor extracts is that GTFP is caused by an agent, such as a viroid (infectious nucleic acid) or prion (infectious protein) (Cohen et al., 1994), that is not detectable by electron microscopy.

Variation in Transmission Success

The 1993 study was designed as a 4 by 5 matrix that assigned one turtle from each of 4 clutches into 1 of 4 experimental groups and one control group. This was done to minimize the chances of transmission failure due to donor factors, such as the stage of tumor progression and the infectious dose of the putative agent, or recipient factors, such as innate or acquired resistance and latent infection. Clutch mates were siblings or half-siblings and therefore more likely to share heritable disease susceptibility factors or prior exposure to GTFP, e.g. vertical disease transmission from the mother. The fact that turtles from all 4 clutches developed GTFP indicated that all the experimental turtles were probably susceptible. Transmission success varied between fibropapilloma preparations, however, since the tumor homogenate prepared from donor #4 failed to induce fibropapillomas in any recipients. The tumors collected from

donor #4 may have been at the wrong developmental stage for infectious particle production or the infectious dose may have been too low. For example, in bovine cutaneous fibropapillomas there is a prepatent period during which productive papillomavirus infection cannot be detected (Olson et al., 1992). In poikilothermic animals such as the green turtle, exogenous factors may influence virus production and shedding. For example, in herpesvirus induced Lucké renal adenocarcinoma of leopard frogs, infectious tumors are only found at the low temperatures required for the production and shedding of virus. Higher temperatures result in rapid growth and metastasis of non infectious tumors (McKinnell, 1981, 1984; McKinnell & Ellis, 1972; Zambernard & Vatter, 1966).

Environmental Influences on Tumor Development

The development of detectable tumors in experimental turtles showed a lag time ranging from 15 to 43 weeks. Although, in the 1993 study, two sets of replicate experiments were conducted 8 weeks apart, most tumors developed concurrently. An exogenous factor such as season or water temperature may have helped to synchronize tumor development. Temperature effects on the development and growth rates of neoplasia have been well-documented in poikilotherms (Asashima et al., 1985; Bowser et al., 1990b; Brown et al., 1976). While these data are not conclusive, future studies should examine the effects of different environmental temperatures on the efficiency of disease

transmission and tumor development. The time lag for development of tumors caused by oncogenic viruses may also be influenced by infectious dose (Beard et al., 1955). In this study it was not possible to measure or control the dose of infectious agent in the various tumor extracts.

Conclusion

Taken together, these studies are the first to demonstrate that GTFP is caused by a filterable infectious agent. Efforts to isolate and test the GTFP-associated herpesvirus, and to identify, isolate, and test other virions and viral genomic sequences from transmission positive tumor extracts must continue in order to fulfill Koch's postulates for this disease.

CHAPTER 5
INITIAL ATTEMPTS TO ISOLATE AND CHARACTERIZE THE ETIOLOGIC
AGENT

Introduction

Controlled transmission experiments (Chapter 4) have demonstrated that green turtle fibropapillomatosis (GTFP) is caused by a filterable infectious agent. The GTFP-associated herpesvirus identified in some experimentally induced tumors may be the cause of GTFP but, until Koch's postulates can be fulfilled using this virus, it is but one of several possible viral etiologies (see Chapters 2 & 4).

This chapter describes a series of experiments aimed at isolating the GTFP-associated herpesvirus and other candidate viruses. First, attempts were made to culture the GTFP-associated herpesvirus. Successful cultivation of the herpesvirus would allow experiments to be conducted to fulfill Koch's postulates, and also provide viral antigens and nucleic acids for diagnostic test development. Second, attempts were made to purify the GTFP agent directly from transmission positive tumor homogenates using isopycnic gradient ultracentrifugation. Purification of the agent directly from infectious tumor homogenates would provide virus for further experimentation in the event that the virus could not be cultured. Third, modified transmission

experiments were conducted to provide some basic information about the characteristics of the GTFP agent that are necessary for infectivity, thereby providing additional clues to its identity.

The transmission experiments described below tested the following hypotheses about the GTFP agent: 1) the agent has a lipid containing envelope, required for infectivity, that is destroyed by organic solvent extraction, 2) the infectious agent is a particle (virion), large enough to be cleared from solution by ultracentrifugation, rather than an infectious nucleic acid (viroid, plasmid, or genome fragment) and 3) the agent is hardy enough to survive long-term freezer storage.

Materials and Methods

Virus Isolation in Cell Culture

Frozen tumor extracts from 6 transmission study donors and from 3 turtles with experimentally induced tumors, that were shown by histopathology to have herpesvirus-like inclusions, were used to inoculate subconfluent cultures of TH-1 Terrapene heart cells purchased from the American Type Culture Collection (ATCC CCL50) or green turtle normal skin fibroblasts (see Chapter 8). Fibroblast cultures were grown in 6 well plates (Costar, Cambridge, MA, USA) in Dulbecco's Modified Eagle Medium with nutrient mixture F-12 (D-MEM/F12, GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) at 30°C in 5% CO₂ atmosphere. For infection, subconfluent cultures were washed with three

changes of serum free media (D-MEM/F12). Crude tumor homogenates prepared as for transmission experiments were diluted (1:10 through 1:500) in Hank's Balanced Salt Solution (HBSS). Aliquots (500-1000 μ l) were applied to each well of target cells and allowed to incubate for 30-60 minutes at 30°C to allow for attachment and cell entry. The wells were then filled with serum-free media and allowed to incubate overnight. Infected cultures were then incubated for up to 3 weeks in media supplemented with 10% FBS and monitored for cytopathic effects.

Virus Extraction from Tumor Homogenates

The transmission positive tumor pool from 1993 transmission study donor #3 (Pappy) was used in an attempt to purify enough of the infectious agent by isopycnic gradient ultracentrifugation to use in a transmission experiment. The protocol was based on that used to extract papillomaviruses from tumors (Lancaster et al., 1976). Briefly, frozen aliquots of cell-free tumor extract and the centrifuge pellets (20 g) that resulted from the clarification of crude tumor homogenate were thawed and suspended in 80 ml TNE buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5). This material was homogenized for 10 minutes in a Tissuemizer (Tekmar, Cincinnati, OH, USA) on ice. This material was then centrifuged at 12,000 rpm (10,000 x g) in a Marathon high speed centrifuge for 15 minutes at 4°C and the supernatant saved. The pellet was resuspended in 60 ml TNE to which 10%

Sarkosyl (N-lauroylsarcosine) to a final concentration of 0.15% and 40 ml Freon (1,1,2 trichloro-trifluoroethane) were added. This material was homogenized on ice for 10 minutes and then centrifuged for 15 minutes at $(10,000 \times g)$ at 4°C . The aqueous phase was added to the previous supernatant and the pellet and organic phase were discarded. The pooled supernatant was divided into 4 tubes and centrifuged at 28,000 rpm $(141,000 \times g)$ in a Beckman SW28 rotor for 2 hours at 4°C . The supernatants were discarded. One pellet was fixed in 2% glutaraldehyde for electron microscopic examination. The remaining three pellets were combined and resuspended in 3 ml of TNE by stirring overnight at 4°C .

Isopycnic gradient. The pelleted material, resuspended in TNE, was combined with saturated cesium chloride to a final concentration of 37.8% v/v CsCl, having a refractive index of 1.37 and a density of 1.38 g/ml. This was centrifuged for 24 hrs at 40,000 rpm $(150,000 \times g)$ at room temperature in a Beckman SW 50.1 rotor. Fractions (0.5 ml each) were collected from the top of the gradient by displacing the gradient from the bottom with saturated CsCl. The refractive index and optical density at 280 nm of each fraction were monitored. Fractions of interest (ranging in density from 1.31 to 1.37 g/ml) were pooled and diluted in TNE, centrifuged for 2 hours at 40,000 rpm $(150,000 \times g)$ in SW50.1 rotor. The pellet was resuspended in 1 ml TNE and aliquots were adsorbed on carbon coated 400 mesh copper grids

stained with uranyl acetate and examined by electron microscopy. An aliquot of this material was dialyzed into PBS and a total of 600 μ l was injected intradermally into one green turtle that had been a control in the 1993 transmission study.

Characterization Experiments

Materials for inoculation

In lieu of purified virus with which to conduct experiments, the following characterization experiments used filtered cell-free tumor homogenates.

Donor turtles. Seven free-ranging juvenile green turtles (34-56 cm SCL) with GTFP that stranded or were collected in Florida between August and December 1994 were used as tumor donors (Table 5-1). Each had multiple cutaneous fibropapillomas ranging in size from a few millimeters to over 22 cm in diameter involving the axillary and inguinal regions, flippers, neck, and eyes. Three turtles, N5218, Billy, and Muddy subsequently died. In addition, frozen aliquots of tumor homogenates from two 1993 transmission study donors, Flamingo (donor #1) and Everglades (donor #2) were pooled and used.

Tumor collection. Donor turtles were anesthetized, and all cutaneous tumors were excised as previously described (Chapter 4). Representative tumor sections were fixed in neutral buffered 10% formalin for histopathologic examination. The remaining material was placed in a sterile

Table 5-1. Free-ranging green turtles with cutaneous fibropapillomatosis used as fibropapilloma donors

Identity	Recovery Date ^a	Location (Florida, USA)	SCL (cm)	Wet Weight of Tumor Pool (g)	Storage Duration ^b (days)
N5218	09 Aug 94	Indian River Co., Indian River Lagoon	34.0	39.0	21
N5219	16 Aug 94	Indian River Co., Indian River Lagoon	39.0	53.5	14
Billy	01 Sep 94	Monroe Co., 7 Mile Bridge	55.8	39.0	82
Sunshine (QQR465)	08 Sep 94	Citrus Co., Homosassa	41.0	81.0	88
Mate (QQR466)	12 Sep 94	Monroe Co., Lower Matecumbe Key	53.6	84.0	83
Muddy	29 Nov 94	Monroe Co., N. of Key West	49.2	70.0	0
Carrie (QQJ255)	02 Dec 94	St. Lucie Co., St. Lucie Power Plant	45.2	51.0	0

^a Date of capture or stranding.

^b Length of time that tumors were stored at < -70°C prior to use.

cup, weighed, and placed on dry ice until processed the same day, or stored at -80°C for future use.

Preparation of cell-free tumor extracts. Between 39.0 and 84.0 g of cutaneous fibropapillomas from each donor were used to prepare tumor extracts for use in these experiments (Table 5-1). Two tumor preparations were freshly produced on the same day that they were removed from donors. The remaining tumor pools had been stored at -80°C for 14 to 88 days prior to processing (Table 5-1) and in these cases were prepared 24-48 hrs before inoculation and stored in liquid nitrogen. The pooled material from the 1993 transmission study had been stored for just over 1 year at -180°C prior to use. Pooled fibropapilloma fragments from each donor were thawed and a cell-free twice frozen and thawed 33% (w/v) tumor homogenate prepared as described in chapter 4 for the 1993 study. A portion of each cell-free preparation was filtered through a $0.45\text{ }\mu\text{m}$ syringe tip filter to remove contaminating bacteria and any remaining intact cells. The remaining homogenate, along with the centrifugation pellets and aliquots of filtered homogenate, was frozen in liquid nitrogen and stored at $\leq -80^{\circ}\text{C}$ for future experiments.

Chloroform extraction. Aliquots (5-6 ml) of tumor homogenate or filtered extract were diluted with an equal volume of cold chloroform and vortexed for approximately 5 minutes. After centrifugation at 2000 rpm ($1000 \times g$) in a

Beckman centrifuge for 10 minutes at 4°C, the aqueous phase was decanted and used for injection.

Ultracentrifugation to pellet agent. Samples of filtered tumor homogenate (9 ml total volume) were centrifuged at 34,000 rpm (100,000 x g) for 2 hours at 4°C in a SW50.1 rotor (Beckman) onto a glycerin cushion. The top supernatant was removed, leaving the 1-2 ml of liquid immediately above the pellet, and saved for inoculation. The pellet was resuspended in 0.5 ml of bottom supernatant and used for inoculation. Assuming that the putative GTFP agent was completely cleared from the supernatant, the resuspended pellet had an approximately five-fold higher concentration of agent than the homogenate.

Experimental turtles

Twenty one captive-reared green turtles were used in this study. Five to seven eggs were collected from each of 4 green turtle nests that were deposited by different females within a one week period in August 1993 on Hutchinson Island, Martin County, Florida. None of the nesting females were observed or identified (B. Schroeder, pers. comm.). The protocols for collecting and incubating the eggs and raising hatchlings were the same as previously described (Chapter 4). Turtles (5-6 from each clutch) were between 9 and 13 months old when used in experiments.

Transmission experiments with tumor preparations

The various tumor preparations from each donor were each used to treat 1 to 3 recipient turtles. Treatments included unfiltered homogenate, 0.45 μ m filtered homogenate, chloroform extracted homogenate, ultracentrifuge pellet, ultracentrifuge supernatant, and saline (control), administered as 200 μ l intradermal injections. Each recipient turtle was injected with all tumor preparations from a single donor. Treatments were given in a fixed sequence starting at a different injection site on each turtle so that treatments were somewhat evenly distributed with respect to anatomic location. Injection sites included the proximal margin of a large scale on the palmar surface of the front flipper, the inguinal area, proximal margin of a large scale on the dorsal surface of the rear flipper, dorsal neck, ventral neck, and shoulders. Eyelids were not treated in these experiments because of the proven difficulty in curing tumors at this site. Three turtles received no injections but were housed with their treated clutch-mates to act as sentinels for spontaneous disease.

Treated turtles were held and monitored as previously described (Chapter 4). Blood samples for health monitoring and plasma banking were collected prior to any experiments and periodically during the monitoring period. Experimentally induced tumors were biopsied and the samples processed for light and electron microscopic examination as previously

described (Chapter 4). As of June 1995 this experiment has been in progress for 7-10 months and monitoring will continue for at least 6 more months.

Results

Virus Culture

Several preliminary attempts to culture the GTFP-associated herpesvirus from experimentally induced tumors or transmission study donor preparations on cultured fibroblast lines (TH-1 or GT) were unsuccessful. No discernible cytopathic effects could be detected at 25 or 30°C. Many cultures became contaminated with fungus.

Virus Purification from Tumor Homogenate

The pooled CsCl fractions spanned the density range 1.31-1.37 g/ml. Negative staining electron microscopy of this sample revealed the presence of numerous round particles of various sizes (Figure 5-1). One particle type was approximately 90-100 nm and superficially resembled intact herpesvirus (Spring & Roizman, 1968; Roizman & Furlong, 1974), but the morphology was not diagnostic (Figure 5-1A, B). The other type of particle was smaller, measuring approximately 50-60 nm and appeared to have a dense center surrounded by a halo that was penetrated by the negative stain (Figure 5-1C). These particles resembled the nucleocapsids and core components of herpesvirus in various stages of disruption (Spring & Roizman, 1968). Particles consistent in size and morphology with papillomavirus were

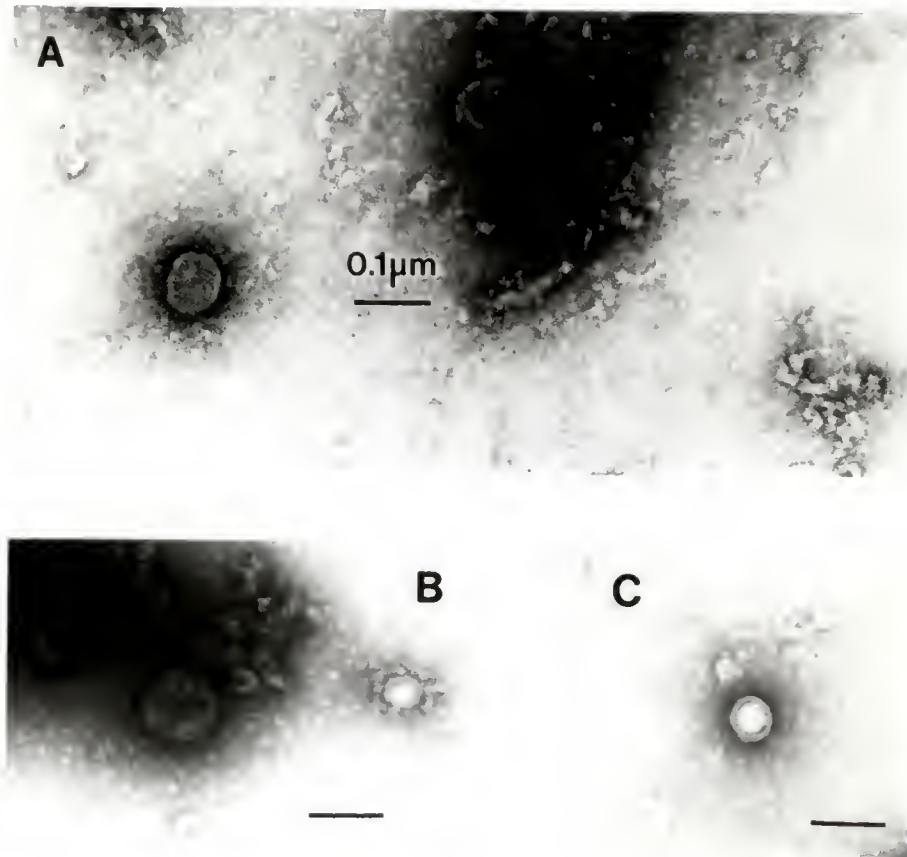


Figure 5-1. Subcellular particles identified in pooled isopycnic gradient fractions prepared from transmission-positive filtered tumor homogenate. Particles in filtered tumor homogenate from donor #3 (1993 transmission study) were separated by ultracentrifugation in a CsCl gradient and the gradient fractions covering the density range 1.31-1.37 g/ml were pooled and examined by negative staining electron microscopy (2% uranyl acetate). Large particles 90-100 nm (A & B) resemble complete herpesvirions although distinct nucleocapsid morphology was not recognized. Smaller particles, approximately 50-60 nm (C) resemble nucleocapsids in various stages of disruption but are not diagnostic. Particles resembling papillomaviruses were not found. (X 100,000).

not found. The turtle that was injected with this CsCl fraction pool has not developed tumors at any site, as of June 1995 (10 months post-inoculation).

Characterization Experiments

Transmission experiments were conducted on the following dates: 30 August 1994 (using homogenates from donors N5218, N5219, and pooled material from Everglades and Flamingo), and 5-6 December 1994 (using homogenates from the remaining donors). Preliminary results of these experiments were tabulated in June 1995 (Table 5-2), after 10 and 7 months of monitoring, respectively. Overall, tumors developed at one or more inoculation sites in 14 recipient turtles. The three control (sentinel) turtles did not develop spontaneous tumors during this period. Tumors did not develop at saline inoculated sites or at any uninoculated sites. The tumor preparation from 1 donor (Sunshine) did not cause tumors in the one recipient that was tested.

Storage (freezer) sensitivity. Five tumor pools used in this study had been stored for 14 to 88 days at -80°C . Four of these preparations successfully induced tumors. The unsuccessful preparation had been stored the longest (88 days) but 2 successful preparations had been stored nearly as long (82-83 days). Similarly, the pooled donor 1 and 2 preparation that had been stored for 1 year at -150°C , thawed and refrozen was also still infectious.

Table 5-2. Summary of characterization transmission experiments.

Donor	No. of Recipients	Saline ^a	Untreated Prep ^b	Ultracentrifuge Prep	
				Chloroform	Pellet Supernatant
1&2 pool	3	0	3	0	nt ^c nt
Sunshine	1	0	0	0	nt nt
Muddy	2	0	2	0	nt nt
Carrie	2	0	2	0	nt nt
N5218	3	0	3	0	0 0
N5219	3	0	1	0	0 0
Mate	2	0	2	0	2 0
Billy	2	0	1	0	0 0
Controls	3	nt	nt	nt	nt nt

^a Data are the number of turtles that developed tumors in at least one inoculation site in each treatment group. Results as of 21 June 1995.

^b Untreated preparations were either 0.45 µm filtrates or unfiltered homogenates.

^c Not tested. No tumors developed at uninoculated sites.

Chloroform sensitivity. Table 5-3 compares the frequencies of fibropapilloma development among turtles inoculated with chloroform-extracted and untreated (unfiltered or filtered) tumor preparations. None of the 18 recipient turtles developed tumors at any site that was inoculated with chloroform extracted tumor preparations. In contrast, 14 (77.8%) of the 18 turtles developed tumors at one or more site that were inoculated with filtered or unfiltered tumor homogenates. The difference in relative frequencies of success was statistically significant (Chi-Square = 22.9, $p < 0.001$).

Ultracentrifugation. Table 5-4 compares the frequencies of tumor induction in recipient turtles between untreated homogenates, ultracentrifuge pellets, and ultracentrifuge supernatants. Treatment with filtered tumor homogenates resulted in tumor induction in 7 (70%) of the recipients. The ultracentrifuge pellets induced tumors in only 2 turtles and supernatants did not induce tumor formation in any turtles. There was a statistically significant difference in the frequency of success among various preparations (Chi-Square = 12.4, $p < 0.005$).

Time to tumor development. Earliest indications of tumor development were identified as slightly raised epidermal swellings ranging from 0.5 to 5.0 mm maximum diameter. The earliest lesions were detected at 7 weeks post inoculation in

Table 5-3. The effect of chloroform treatment on the infectivity of the GTFP agent

Donor	Treatment ^a	
	Untreated Homogenate	Chloroform Extract
1&2 pool	3/3	0/3
Sunshine	0/1	0/1
Muddy	2/2	0/2
Carrie	2/2	0/2
N5218	3/3	0/3
N5219	1/3	0/3
Mate	2/2	0/2
Billy	1/2	0/2
Total	14/18	0/18

^a Number of turtles that developed tumors in at least one inoculation site over the number of turtles inoculated. Results as of 21 June 1995.

Table 5-4. Partitioning of GTFP agent infectivity by ultracentrifugation

Donor	Treatment ^a		
	Filtered Homogenate	Ultra-centrifuge Pellet	Ultra-centrifuge Supernatant
N5218	3/3	0/3	0/3
N5219	1/3	0/3	0/3
Mate	2/2	2/2	0/2
Billy	1/2	0/2	0/2
Total	7/10	2/10	0/10

^a Number of turtles that developed tumors in at least one inoculation site over the number of turtles that were treated. Results as of 21 June 1995.

three turtles (E-3, E-4, and E-5 inoculated in December 94) but these did not progress for several months. Additional early tumors were first detected in April 95, between 20 to 28 weeks after inoculation comparable to the lag time reported from earlier experiments (Chapter 4).

Light and electron microscopy. Experimentally induced fibropapillomas were consistent with those previously described (Chapter 4). These biopsies have not yet been examined by electron microscopy for evidence of virus.

Discussion

This study attempted to isolate the GTFP-associated herpesvirus and other potential GTFP agent(s) and began to describe the characteristics of the GTFP agent that are associated with infectivity.

Virus Culture

Failure to culture the GTFP-associated herpesvirus on two turtle cell lines could be due to several factors. One possibility is that the cell lines or culture conditions used were not permissive for virus entry into cells or virus replication following entry. Because the GTFP-associated herpesvirus causes histologically detectable cytolytic changes in situ only in tumor epidermal cells and may cause proliferation of histologically normal fibroblasts, it is possible that cytopathic effects would not be observed in cultured fibroblasts even if they were infected. A second possibility is that there was insufficient infectious virus

within the preparations that were tested. A third possibility is that the prepatent period of the virus was longer than the 2-3 week observation period.

Attempts by others to culture the GTFP-associated herpesvirus have also failed (Gail Sherba, University of Illinois, Urbana, IL 61801, pers. comm.). Future attempts at culturing this virus should use epithelial cell lines since these are known to be productively infected in the natural disease. These results, while disappointing and inconclusive, lend support to the hypothesis that this is a new herpesvirus of green turtles because both the herpesvirus of gray-patch disease (Rebell et al., 1975) and the herpesvirus associated with respiratory disease in green turtles (Jacobson et al., 1986a) were culturable in reptilian fibroblasts under conditions similar to those used in this study.

Virus Purification

Purification of virus particles directly from experimentally proven transmission positive tumor preparations was attempted as an alternative to virus culture because the conditions necessary for in vitro propagation of the GTFP-agent were unknown and may require extensive time and experimentation to discover. It is also possible that, like the papillomaviruses (Meyers et al., 1992; Shah & Howley, 1990), the GTFP agent may not be culturable using standard virus culture techniques. In addition, purification of virus directly from tumors potentially allows the

identification of all viruses present. Because a papillomavirus could still be the cause of GTFP (see Chapter 4) this approach made it possible to determine the presence of papillomavirus. In fact the protocol used was originally designed to concentrate and purify papillomaviruses (Lancaster et al., 1976). Most unenveloped viruses and de-enveloped nucleocapsids will concentrate within a narrow range of densities in a CsCl gradient (1.27-1.45 g/ml), providing an opportunity to isolate any particle type (Fenner et al., 1974; Klingeborn & Pertoft, 1972).

Examination of pooled fractions covering the density range (1.31-1.37 g/ml) by negative staining electron microscopy revealed the presence of particles with morphologic features consistent with various forms of herpesvirus but these features were not diagnostic. The extraction procedure used is known to damage enveloped viruses and this may explain the poor morphologic quality of particles and the range of putative virion components observed (Spring & Roizman, 1967; 1968). On the other hand, this extraction procedure does not damage unenveloped viruses and, had sufficient numbers of papillomavirions (density = 1.34 g/ml) been present, they should have been easily recognized (Lancaster et al., 1976). The absence of detectable papillomavirus particles in GTFP extracts, combined with the absence of detectable papillomavirus antigens and gene sequences (Jacobson et al., 1989; see

Chapter 2) supports the hypothesis that GTFP is caused by a virus other than papillomavirus.

Transmission of GTFP to a single recipient using the CsCl gradient fraction pool containing putative herpesvirus-like particles was unsuccessful. Thus, the identity of these particles as well as their role in disease, remains in question. Although, it is possible that these particles are artifacts, e.g. membrane vesicles, they could be virus components that lost infectivity during the purification process, i.e. detergent and freon extraction, CsCl exposure. Infectivity of herpesviruses is known to be lost under these conditions whereas the infectivity of unenveloped viruses is not (Spring & Roizman, 1967). Thus, one would not expect transmission to be successful if the GTFP associated herpesvirus is the etiologic agent. On the other hand, transmission should have been successful if papillomavirus was the agent. Further attempts to purify the GTFP agent from infective tumor extracts should try to preserve its infectivity (Valne & Blomberg, 1974). Even if infectivity is lost, however, gradient purified virus containing fractions can be used as a source of viral genomes for cloning and sequencing and viral antigen for use in immunodiagnostic assays.

Characterization Experiments

Among enveloped viruses, such as herpesviruses, poxviruses, and retroviruses, the viral envelope is important

for virion stability in the environment and for efficient attachment and entry into cells (early infection), although cells may also become infected via pinocytosis of nucleocapsids (Howe et al., 1980; Roizman & Furlong, 1974). If an enveloped virus such as the GTFP-associated herpesvirus is the cause of GTFP, one would expect transmission success (infectivity) to be greatly reduced by treatment of GTFP homogenates with detergents or organic solvents, such as chloroform, that disrupt the viral envelope (Fenner et al., 1974). On the other hand, if an unenveloped virus, such as a papillomavirus, is the cause of GTFP, it should remain infectious following treatment, and transmission success should not be reduced. Preliminary results of transmission experiments, 7-10 months after inoculation, indicate that the GTFP agent is chloroform sensitive because tumors did not develop at any sites that were inoculated with chloroform-extracted tumor preparations, whereas tumors were produced at sites that were inoculated with untreated homogenates. These results are consistent with the hypothesis that GTFP is caused by an enveloped virus.

The infectivity of enveloped viruses may also be destroyed by frequent freezing and thawing and prolonged storage, although most viruses are stable at -70°C or below (Fenner et al., 1974). The fact that the GTFP agent remained infective after being held for nearly 3 months at -80°C or for a year at -150°C shows that it is not extremely sensitive

to long-term storage and thawing, and that, potentially, it can be purified in fully infectious form from archived frozen tumors and tumor homogenates. This opens the possibility for future experiments using extracts, whose effective (infectious) dose has been titrated, to test the sensitivity of the GTFP agent to physical and chemical factors such as temperature, desiccation, pH, osmolarity, disinfectants, and detergents.

Previous experiments (Chapter 4) showed that the GTFP agent passed through a 0.45 μm filter but was retained by a 0.2 μm filter. Thus, the agent is either a relatively large particle or is tightly cell-associated. The only particles observed in tumors or their extracts have been morphologically consistent with herpesvirus. However, the possibility that the GTFP agent is another type of virus, that does not replicate in tumors but is present as infectious DNA or RNA, must be eliminated.

Ultracentrifugation was used to partition infectivity between pellet and supernatant based on the agent's sedimentation coefficient. According to the manufacturer's specifications for the SW50.1 rotor (Beckman, Palo Alto, CA, USA), a 2 hour run at 34,000 rpm should effectively pellet all particles > 64 Svedberg Units (S). Sedimentation coefficients for viruses range between 40 and several thousand S. The largest DNA particles have sedimentation coefficients ≤ 100 S. Although infective DNA may have been

bound to and sedimented with cellular debris, this experiment demonstrated that the agent could be concentrated by pelleting in an ultracentrifuge, which can be used as a preliminary step in a scheme to purify the agent or its genome. Although the pellets should have contained higher concentrations of virus particles than the starting tumor homogenates, pellets were less successful at inducing experimental tumors than unprocessed homogenates. This suggests that the ultracentrifugation conditions (speed, time, buffer) may have damaged virus particles. This apparent sensitivity to conditions provides another clue that the GTFP agent may be a large enveloped virus, such as the GTFP-associated herpesvirus.

CHAPTER 6
HISTOPATHOLOGIC AND IMMUNOHISTOCHEMICAL EVIDENCE FOR A VIRAL
(HERPESVIRUS) ETIOLOGY

Introduction

The studies presented in chapters 4 and 5 showed that GTFP is caused by an infectious agent with a sedimentation coefficient greater than 64 S, a functional diameter $< 0.45 \mu\text{m}$, and which is sensitive to chloroform. Koch's postulates demand that the GTFP agent must be present in diseased tissues of the donor as well as in lesions experimentally produced in recipients. So far, the herpesvirus identified has been found in both experimentally induced (Chapter 4) and in spontaneous tumors (Jacobson et al., 1991) fulfilling these two criteria. In addition to identifying the putative agent in tumors, the proposed pathogenesis should account for all the major histologic features seen in spontaneous lesions and should be consistent with what is known about closely related pathogens.

This chapter presents histopathologic descriptions of spontaneous GTFP from a series of free-ranging turtles from 2 geographically separate populations and an analysis of the associations among various epidemiologic and histologic features. In addition, the histologic features of a series of known-age experimentally induced tumors were compared with

spontaneous lesions to help distinguish the relevant pathologic processes from incidental findings and to provide a preliminary description of GTFP progression.

Special procedures, such as immunofluorescence staining of tumors for antibody complex deposition and immunohistochemical screening for herpesvirus antigens, were used to test specific hypotheses about the pathogenesis of certain histologic features observed in tumors.

Materials and Methods

Histopathology

Cutaneous fibropapillomas. Fibropapillomas were collected from 25 green turtles that stranded or were captured in Florida waters (Florida Bay, Florida Keys, or Indian River), 15 green turtles from the Hawaiian islands, and 11 captive-reared turtles with experimentally induced GTFP. Multiple tumors were biopsied from each individual under local or general anesthesia by 6 mm punch or surgical excision. Biopsies were fixed by immersion in neutral buffered 10% formalin. After embedding in paraffin, 6 μ m thick sections were prepared, stained with hematoxylin and eosin (H&E), and examined by light microscopy.

Prior to surgery, fibropapillomas were examined grossly to determine if they were pigmented and primarily verrucous or smooth. A minimum of 3 sections were prepared from each tumor. The sections were scored for the presence or absence of epithelial hyperplasia (acanthosis, orthokeratosis),

epithelial integrity (ulceration, focal cellular degenerative changes, and vesicle formation), inflammation (granulocytes, mononuclear cell infiltrate, foreign body granulomas), and potential pathogens (virus inclusions, spirorchid trematode ova, bacteria, fungi, and epibionts). Statistical associations of selected histologic features were determined by Chi-Square analysis.

Visceral tumors. Visceral tumors were found at necropsy in 9 Florida turtles and 1 Hawaiian turtle. These were collected and processed for histological examination also.

Immunohistochemistry for Detection of Herpesvirus Antigens

Antiserum. All biopsies of cutaneous tumors were screened by immunohistochemistry for evidence of herpesvirus antigens using a herpesvirus-specific turtle antiserum. This antiserum (plasma) was collected from a green turtle (TX91-#4) used as a recipient in the first (1991) transmission study (Chapter 4). This turtle had an experimentally induced fibropapilloma for nearly 1 year and subsequently developed additional tumors at other cutaneous sites. Plasma collected from this animal approximately 3 months after multiple tumors developed was tested (see methods below) at various dilutions (from 1:10-1:500 in PBS) on GTFP sections containing eosinophilic intranuclear inclusions that were proven by EM to contain herpesvirus particles. This plasma showed specific intranuclear reactivity within foci of ballooning epidermal

degeneration corresponding to areas containing herpesvirus inclusions (Figure 6-1).

Screening protocol. Single 6 μm sections were cut from paraffin embedded tissue blocks and mounted on silanized glass slides. Sections were deparaffinized in 3 changes of xylene and rehydrated through an alcohol series. Endogenous peroxidase activity was quenched by incubating slides in 3% H_2O_2 for 10 minutes. Antigenicity was recovered by incubation in an enzyme solution (0.125% trypsin, 0.1% CaCl_2 in PBS; pH 7.4) for 20 minutes at 37°C. Slides were then washed for 30 minutes in three changes of PBS and blotted dry. Slides were then flooded with the anti-herpesviral inclusion body antiserum diluted 1:50 in PBS and incubated overnight at room temperature in a humidified chamber. Following incubation with antisera, the sections were washed for 30 minutes in 3 changes of PBS, blotted, and incubated with biotinylated secondary antibody solution for 1 hour. The secondary antibody was a mixture of biotinylated monoclonal antibodies HL673 (1 $\mu\text{g}/\text{ml}$) and HL857 (1 $\mu\text{g}/\text{ml}$) specific for green turtle immunoglobulin light chain and 7S IgY heavy chain respectively (see Chapter 3) in PBS with added normal mouse serum (1:20 dilution). The sections were washed for 30 minutes in 3 changes of PBS, blotted, and incubated for 1 hour with horseradish peroxidase conjugated streptavidin (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:300 in

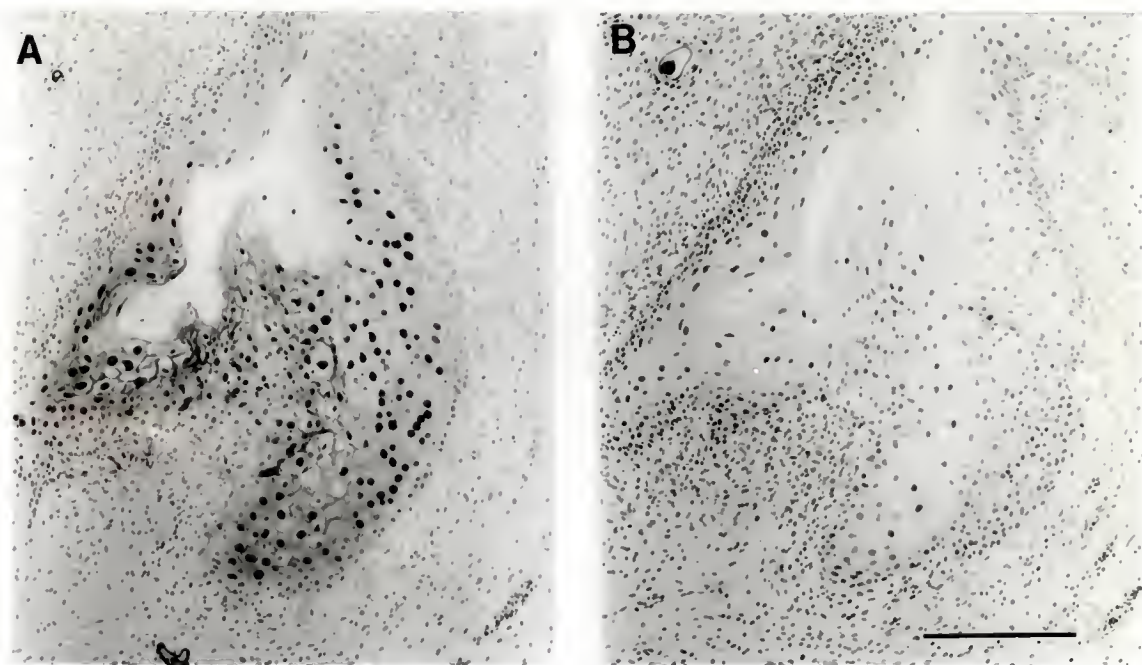


Figure 6-1. Detection of herpesvirus antigens in green turtle fibropapillomas by immunohistochemistry. Spontaneous and experimentally induced tumor sections were screened using antiserum (plasma) from a green turtle (TX91-#4) that had disseminated, experimentally induced GTFP. Plasma from this turtle showed specific immunoreactivity with intranuclear inclusions that had been proven by electron microscopy to contain herpesvirus. Green turtle antibody binding was detected with a mixture of biotinylated Mabs HL673 and HL858, specific for light chain and 7S IgY respectively, and horseradish peroxidase conjugated streptavidin. (A) Herpesvirus-positive GTFP tissue section tested with plasma from TX91-#4 (diluted 1:50), showing positive intranuclear immunoreactivity confined to epidermal cells undergoing ballooning degenerative changes. (B) Herpesvirus-positive GTFP tissue section (same area) tested with normal green turtle plasma (diluted 1:10), showing negative immunoreactivity. Diaminobenzidine substrate counterstained with Harris' hematoxylin (scale bar = 200 μ m).

PBS. After a final wash procedure the slides were immersed in substrate (3,3 diaminobenzidine (Sigma) 500 µg/ml in ice cold PBS) and incubated for 2-10 minutes. The color reaction was monitored in control slides. Sections were counter-stained with Harris's hematoxylin, dehydrated, and mounted.

Immunofluorescence Staining for Immunoglobulin Deposition

A specific hypothesis about the role of immune complexes in the pathogenesis of GTFP was tested by direct immunofluorescence staining. Frozen sections from 2 cutaneous tumors shown to contain separations of epidermis from underlying dermis (clefts) were cut, fixed in acetone for 10 minutes, and incubated for 30 minutes with one of the following monoclonal antibodies to green turtle immunoglobulins: HL857 (7S IgY specific), HL814 (5.7S IgY specific), or HL846 (IgM specific), diluted 1µg/ml in PBS with 1% BSA. Slides were then washed for 30 minutes in 3 changes of PBS and incubated with FITC-conjugated sheep anti-mouse Fab' (Sigma) diluted 1:1000 in PBS for 30 minutes. Slides were then washed, mounted, and observed on a fluorescence microscope.

Results

Cutaneous Fibropapillomas of Florida and Hawaiian Turtles

Biopsies were collected from 119 skin tumors from 25 Florida green turtles captured on the east coast of Florida or in Florida Bay and the Florida Keys and 53 tumors from 15 Hawaiian green turtles from several different sites by George

Balazs (NMFS, Southwest Fisheries Science Center, Honolulu, HI 96822). The Florida green turtles ranged in size from 26 to > 100 cm SCL ($\bar{x} = 57.3 \pm 20.9$) included 4 nesting adult females. The Hawaiian turtles were all juveniles ranging in size from 46.0 to 88.1 cm SCL ($\bar{x} = 60.0 \pm 13.2$). Between 1 and 12 tumors were sampled from each Hawaiian turtle ($\bar{x} = 3.7 \pm 2.7$) and included biopsies from multiple skin sites including 11 eye tumors. Between 1 and 20 tumors were sampled from each Florida turtle ($\bar{x} = 4.8 \pm 4.5$) and included 12 eye tumors. On average, more sections were examined from each Florida turtle than from each Hawaiian turtle and Florida biopsies were larger.

Table 6-1 summarizes the relative frequencies of various histologic features for the two population samples. Those features that had statistically significant difference relative frequencies between the 2 regions are shown and will be discussed below.

Epidermal folding

Tumors were classified based on gross appearance as either verrucous (highly arborized papillary projections supported by an abundant fibrovascular matrix) or fibromatous (primarily fibrous connective tissue with relatively smooth surface) (see Chapter 2, Figure 2-4). The Florida sample had more grossly verrucous highly arborized tumors (74.6%) than Hawaiian sample (51.1%) and this difference in relative frequency was statistically significant (Chi-Square = 8.24, p

Table 6-1. Comparison of the gross and histologic features of fibropapilloma biopsies from free-ranging Florida and Hawaiian green turtles.

Observations	Relative Frequency (%)		Chi-Square
	Hawaii (N = 53)	Florida (N = 119)	
Gross Examination			
Pigmentation	69.8	47.9	p < 0.01
Epidermal Folding	51.1	74.6	p < 0.005
Histopathology			
Acanthosis	96.2	93.3	ns
Orthokeratosis	73.6	84.9	ns
Basal Cell Degeneration	77.4	90.8	p < 0.025
Dermal-Epidermal Cleft	50.9	54.6	ns
Spinous Layer Degeneration	15.1	25.2	ns
Intraepidermal Pustule	17.0	18.5	ns
Ulceration/Erosion	35.8	38.7	ns
Inflammation			
Granulocytes	32.1	47.9	ns
Foreign Body Granuloma	50.9	40.3	ns
Perivascular lymphocytes	58.5	73.1	ns
Infiltrating Lymphocytes	-	-	-

Table 6-1--continued.

Observations	Relative Frequency (%)		Chi-Square
	Hawaii (N = 53)	Florida (N = 119)	
Potential Pathogens			
Virus-like Inclusions	0	1.7	ns
Bacteria/Prokaryotes	66.0	46.2	p < 0.025
Fungi/Algae	32.1	6.7	p < 0.001
Metazoan Epibionts	18.9	9.2	ns
Spirorchid Ova	50.9	30.3	p < 0.01

< 0.005). However, even relatively smooth fibromas often had extensive areas with some degree of epidermal folding detectable at the microscopic level.

Pigmentation

Tumors were pigmented to various degrees ranging from white or pink to grey-green and black. All pigmented tumors had dendritic cells with long fine processes located scattered throughout the dermis, concentrated in and around small arteries or in the stratum basale and spinosum of the epidermis (Figure 6-2A). Heavily pigmented tumors had these cells in all layers and had free pigment granules scattered in the epidermis, whereas some tumors with unpigmented epidermis had pigment cells concentrated around deep dermal vessels. In most tumors, the pigment was always very black. Normal darkly pigmented skin from some areas also had these type of cells. Green skin had aggregations of dendritic cells located in the superficial dermis that contained a pale gold-green pigment. These pigment cells were observed in very few tumors (Figure 6-2B). The Hawaiian sample contained more tumors that were pigmented at the gross and microscopic levels than the Florida sample and this difference was statistically significant.

Pathologic changes in the dermis

Proliferation of fibroblasts in the papillary dermis was found in all tumors. The dermal portion of tumors contained numerous well-differentiated fibroblasts haphazardly arranged

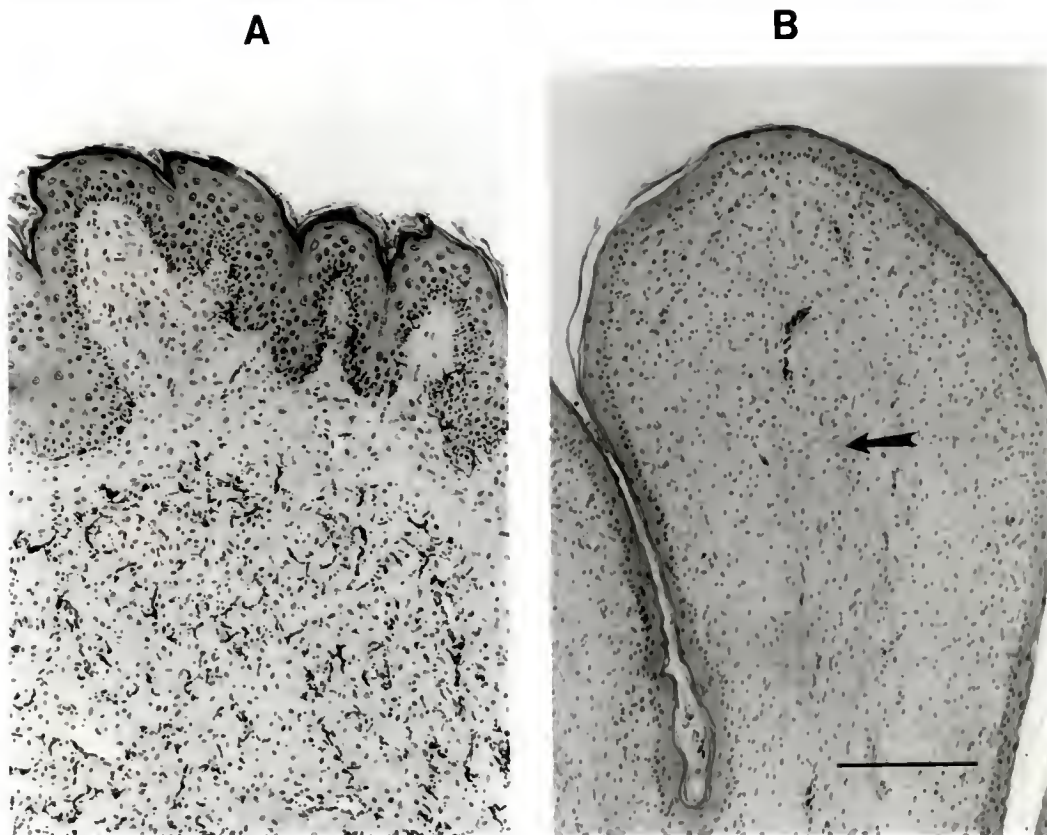


Figure 6-2. Variation in distribution of pigment cells in cutaneous fibropapillomas. (A) Heavily pigmented tumor with dark pigment containing cells in all layers and free pigment within epidermis. (B) Superficially unpigmented tumor with pale gold-green pigment cells distributed in dermis (arrow). H&E (scale bar = 200 μ m).

in a ground substance containing fine collagen bundles. In some tumors the ground substance was more myxomatous than fibrous, especially in the superficial layers, than in others. The superficial dermis was more densely populated with cells than deeper layers. The fibrous portion of tumors could be easily distinguished from deeper reticular dermis (Figure 6-3).

Pathologic changes in the epidermis

A number of epidermal changes were common features in the fibropapillomas. One or more of these features could be found to various degrees in any particular tumor.

Hyperplasia. Epidermal proliferation ranged from minimal (normal skin thickness, 4-7 cells) to extensive (up to 30 cells thick) and consisted of acanthosis and orthokeratosis (Figure 6-4A-D). Acanthosis was observed in about 94% of the biopsies. Orthokeratosis, where the stratum corneum was thickened compared to surrounding normal skin without retention of nuclei, was seen in most tumors (81%), except for those arising from non-cornified epidermis such as the cloacal mucosa and conjunctiva (Figure 6-4D). When these cases are excluded from analysis, orthokeratosis was observed in 89.7% of the biopsies. In several cases hyperkeratosis was extreme and when combined with papillary hyperplasia led to retention of cornified inclusion cysts (Figure 6-4C).

Basal cell degeneration. Vacuolation of basal cell cytoplasm with individual cell necrosis (Figure 6-5A,B) was a

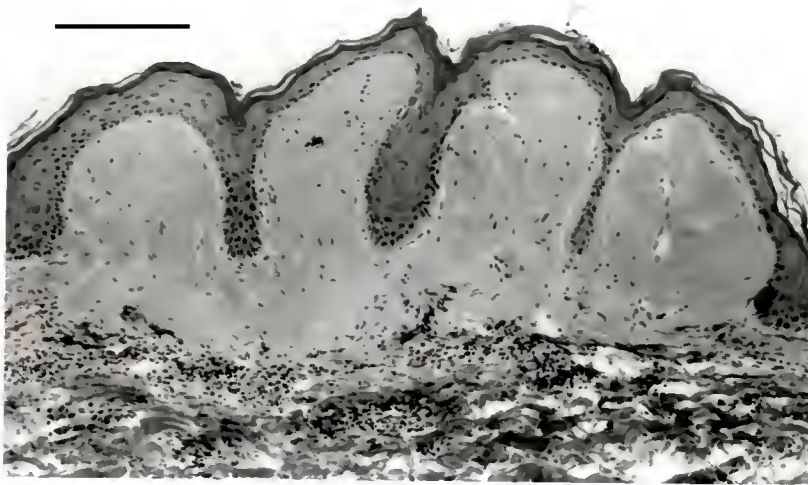


Figure 6-3. Typical cutaneous fibropapilloma in the green turtle. Fibroblast proliferation occurs primarily in the papillary layer of the dermis with the production of fine collagen bundles. Consequently, there is usually a distinct transition from tumor tissue to normal reticular dermis along the deep border of cutaneous fibropapillomas. H&E (scale bar = 200 μm).

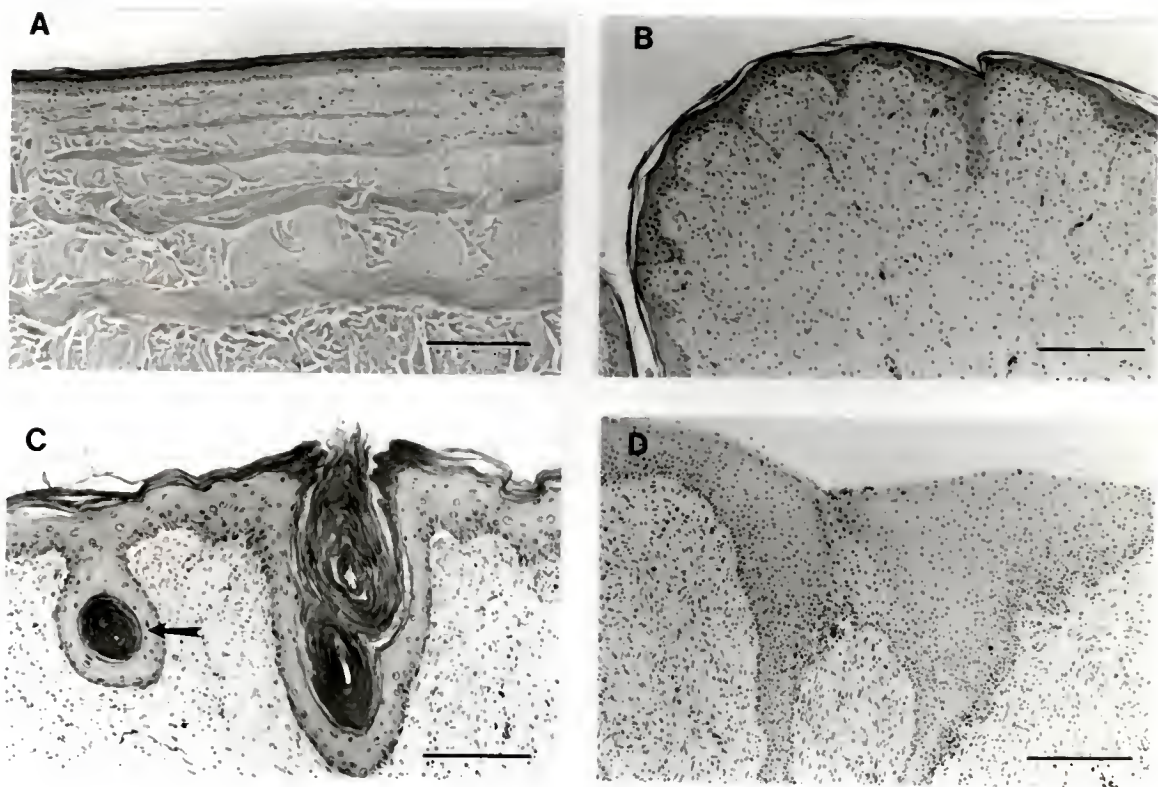


Figure 6-4. Variation in the degree of epidermal hyperplasia among cutaneous fibropapillomas. (A) Normal skin is typically 4-7 cells thick. (B) Cutaneous fibropapilloma with relatively normal epidermis. (C) Cutaneous fibropapilloma showing marked acanthosis and orthokeratotic hyperkeratosis. Note the cornified inclusion cyst (arrow) (D) Conjunctival tumor showing marked epidermal hyperplasia but no orthokeratosis. H&E (scale bars = 200 μ m).

consistent feature and was observed in 86.6% of biopsy sections overall, although significantly more Florida biopsies (90.8%) had this feature than Hawaiian samples (77.4%), (Chi-Square = 5.63, $p < 0.025$). Sometimes, spongiotic changes in the stratum spinosum above locally extensive areas of basal cell degeneration were observed (Figure 6-5A).

Dermal-epidermal clefts. The degenerative changes along the basement membrane resulted in a separation (cleft) between the dermis and epidermis (Figure 6-6 A,C). When this cleft became large, the epidermis above it underwent necrosis and became ulcerated. Cleft formation was observed in over half (53.5%) of the biopsies. There was a statistically significant association of cleft formation with basal cell disruption (Chi-Square = 25.7, $p < 0.001$), but it is not clear if basal cell necrosis is necessary in all cases for cleft formation. Clefts also appeared to form at or below the basement membrane without prior basal cell degeneration (Figure 6-6B). This was confirmed in some sections with Periodic Acid-Schiff (PAS) staining of basement membrane.

Spinous layer degeneration. Sometimes cells in the stratum spinosum above locally extensive areas of basal cell degeneration contained solitary large clear vacuoles that pushed the nucleus to the edge of the cell giving it a crescent shape. Degenerative changes in the stratum spinosum, that were not directly associated with basal layer changes,

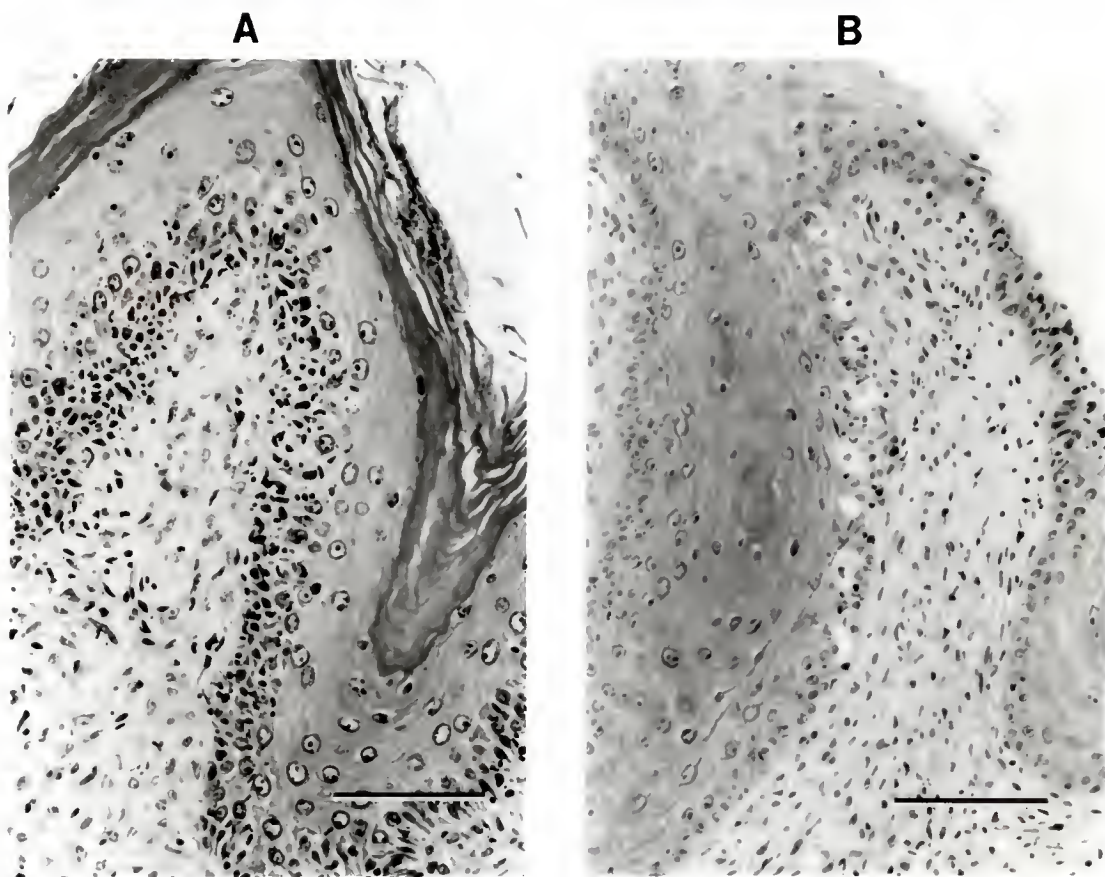


Figure 6-5. Degenerative changes observed in basal epidermal cells of fibropapillomas. (A) Basal cell degeneration accompanied by spongiotic changes in the basal and suprabasal epidermis. (B) Focal ballooning degeneration and necrosis of basal cells. H&E (scale bar = 100 μ m).

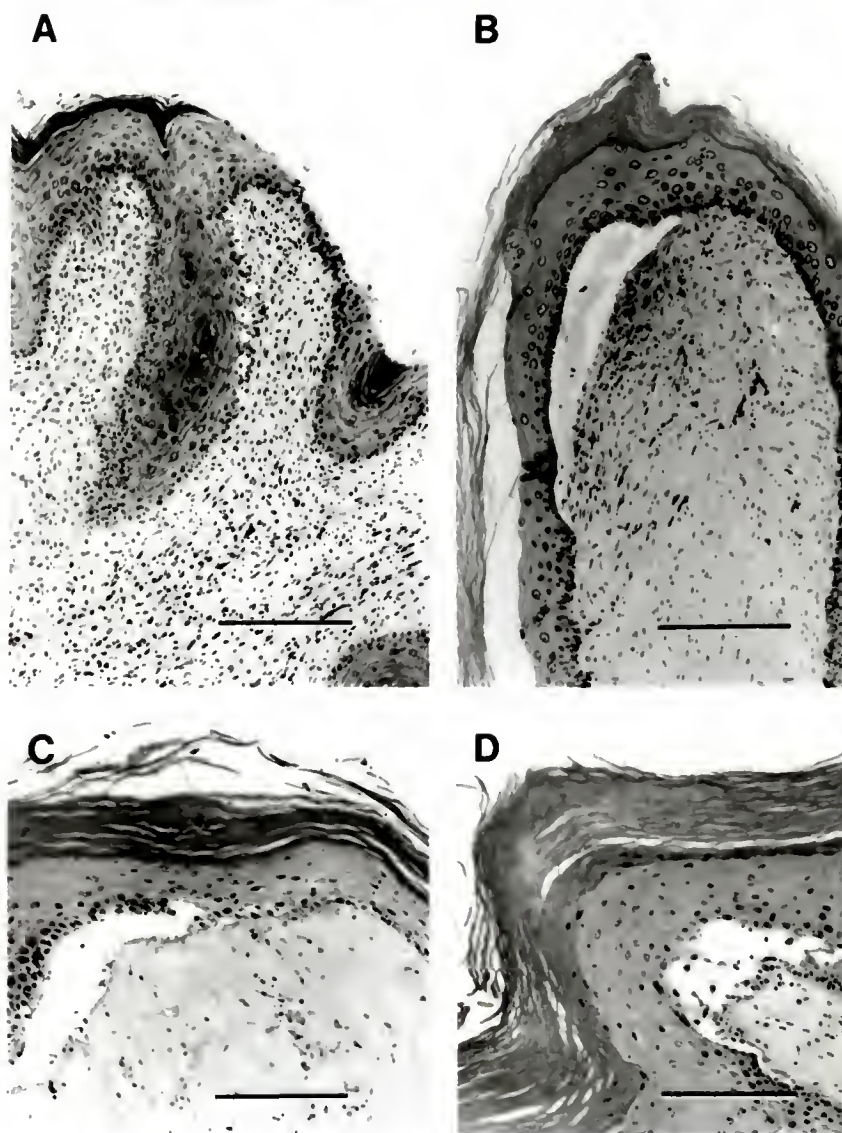


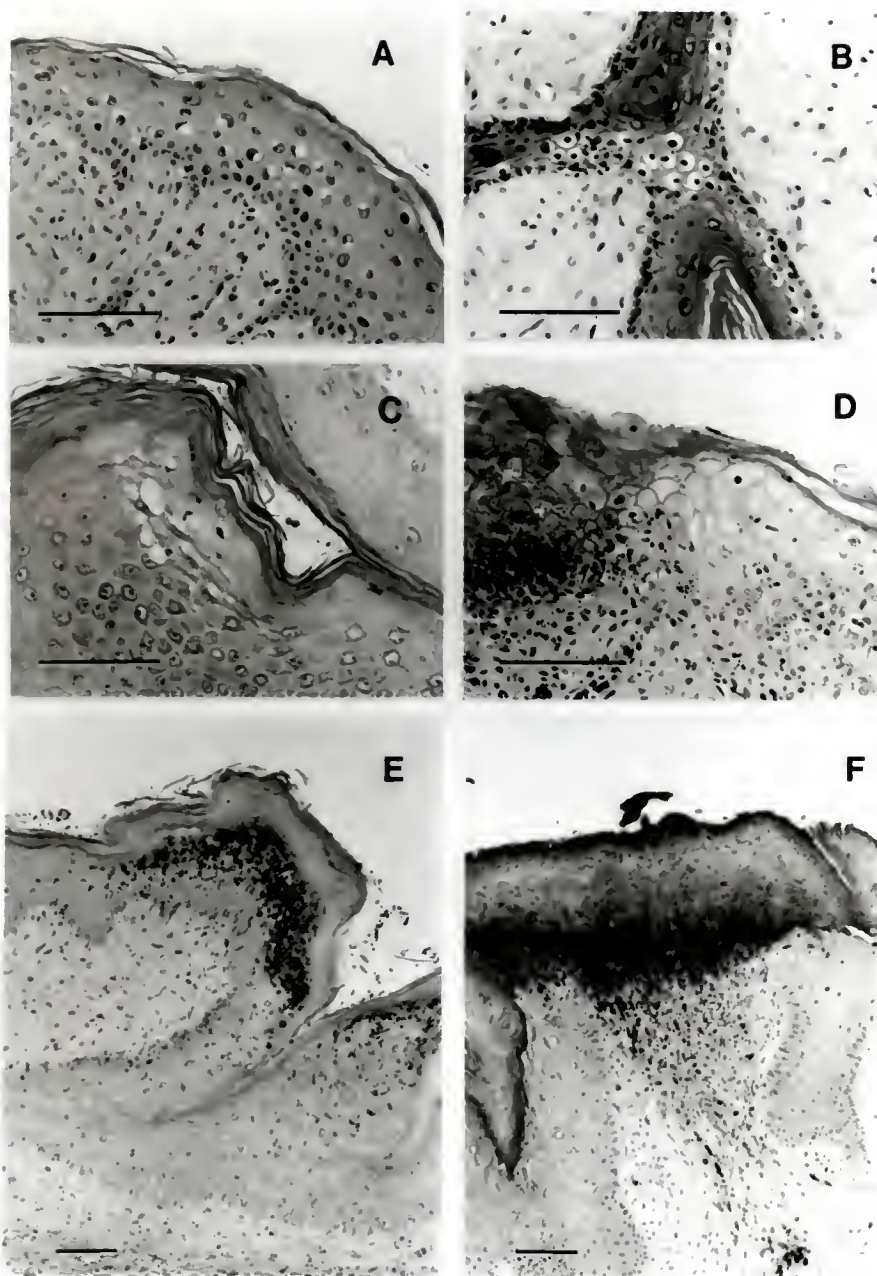
Figure 6-6. Dermal-epidermal clefts in cutaneous fibropapillomas. (A) Early cleft formation associated with basal cell degeneration. (B) Sub-basal cleft formed below the basement membrane with intact basal epidermal cells and eosinophilic material accumulated within the cleft. (C) Cleft formation showing combined features of basal cell degeneration and sub-basal separation. (D) Cleft showing features of sub-basal separation and supra-basal cleft (vesicle) formation. H&E (scale bars = 200 μm).

were also observed (Figure 6-7). These changes ranged from single cell edema leading to perinuclear cytoplasmic clearing (halos) (Figure 6-7B) to more extensive foci of reticular or ballooning degeneration, acantholysis, and intraepidermal vesicle formation (Figure 6-7C,D). In more advanced foci of degeneration and acantholysis, an extensive granulocytic infiltrate led to intraepidermal pustule formation (Figure 6-7E). Foci of severe ballooning degeneration and acantholysis were found in 22% of cases overall. In addition, intraepidermal vesicles and pustules were observed in 18% of tumors. Combined, these progressive pathologic changes were observed in 56 biopsies (32.6%).

Erosion and ulceration. Focal to focally extensive cutaneous erosions and ulcers were observed in about 38% of the biopsies examined and could be attributed to cleft formation at the dermal-epidermal boundary or to degeneration in the stratum spinosum. Erosions and ulcers were heavily infiltrated with granulocytes and were covered by cellular debris and desiccated proteinaceous material (Figure 6-7F).

Taken together, one or more of these epidermal degenerative changes, including basal layer degeneration, spinous layer degeneration, cleft formation, and ulceration, were found in 92% of tumor biopsies examined. The remaining biopsies possibly represent tumors with healed epidermis.

Figure 6-7. Degenerative changes observed in the stratum spinosum of cutaneous fibropapillomas. (A) Sporadic vacuolar degeneration. Single large cytoplasmic vacuoles displace and distort the nucleus. (B) Intracellular edema resulting in perinuclear cytoplasmic clearing (halos). (C) Ballooning degeneration with lysis and intraepidermal vesicle formation. (D) Ballooning degeneration adjacent to an ulcer. (E) Intraepidermal pustule with granulocytic infiltrate accumulating in the upper epidermis below the stratum corneum. (F) Ulcer. H&E (scale bars = 100 μ m).



Inflammation

Three types of inflammatory cell infiltrates were observed in tumor biopsies: foreign body granulomas, granulocytic infiltrates, and round cell (lymphocytic) infiltrates (perivasculitis and dermal infiltrates).

Foreign body granulomas. Small foreign body granulomas were common features, found in 74 (43.0%) of the biopsies from free-ranging turtles. These consisted of multinucleated giant cells with nuclei forming centrifugal chains around the edge of the cell (Figure 6-8). Mononuclear (lymphocytes) were present to various degrees around these areas. Within the area surrounded by giant cells were either spirorchid ova (Figure 6-8A) or cornified inclusion cysts in various stages of degeneration (Figure 6-8B). Spirorchid ova were found more commonly than cornified inclusions, accounting for 85% of the sections that contained foreign body granulomas. One or the other of these two foreign bodies accounted for all granulomas observed.

Granulocytic infiltrates. Granulocytic infiltrates were found in 43% of the biopsies overall. The infiltrates were composed primarily of heterophils, which were large cells containing numerous oval eosinophilic granules. Eosinophils, large cells with clear cytoplasm and relatively few (7-9) large round eosinophilic granules, were also occasionally identified. These cells were difficult to distinguish in sections stained with H&E. Granulocyte margination within

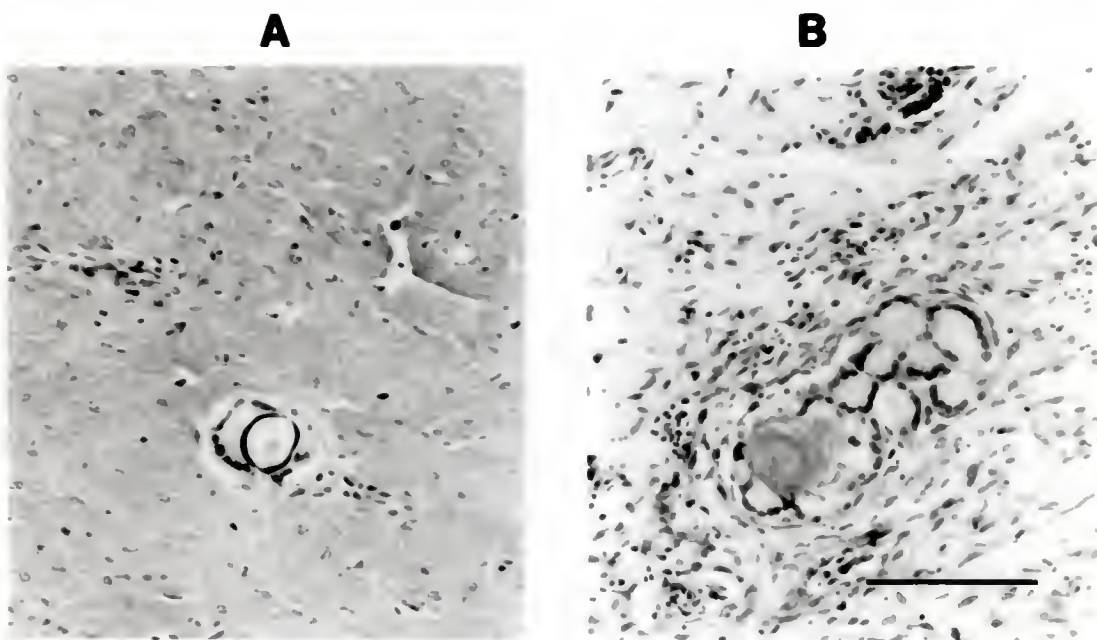


Figure 6-8. Foreign body granulomas found in fibropapillomas of free-ranging green turtles. (A) Multinucleate foreign body giant cell engulfing spirorchid trematode ova. (B) Foreign body giant cell surrounding a cornified inclusion. H&E (scale bar = 100 μ m).

dermal vessels, migration through the tumor, and aggregation were strongly associated (Chi-Square = 12.88, $p < 0.001$) with disruption of the integrity of the epidermis, i.e. erosions, ulcers, or intraepidermal pustules (Figure 6-7E,F). In total, 81% of sections with granulocytic infiltrates also had erosions, ulcers, or pustules. Similarly 86% of sections with erosions or ulcers had obvious granulocytic infiltrates. In contrast, granulocytic infiltration was not associated with cleft formation in otherwise intact epithelium (Chi-Square = 3.73, $p > 0.05$).

Lymphocytic infiltrates. The fibromatous portion of tumors consisted of very dense connective tissue composed of fine collagen bundles that could be easily differentiated from the underlying reticular dermis. Extensive lymphocyte infiltration at the junction of normal and tumor dermis was a common feature in those sections where this area was represented (Figure 6-9A-C). Because many biopsies did not extend deep enough into the dermis, the relative frequency of this type of infiltrate could not be determined.

Small arteries and veins permeated the fibromatous portion of all tumors. There were various degrees of inflammation around vessels, which were graded as follows: 0 = no inflammation, 1 = a few scattered lymphocytes but not enough to surround the vessels in the section, 2 = numerous lymphocytes completely surrounding vessels (Figure 6-9D), and 3 = lymphocytes surrounding vessels and extending into

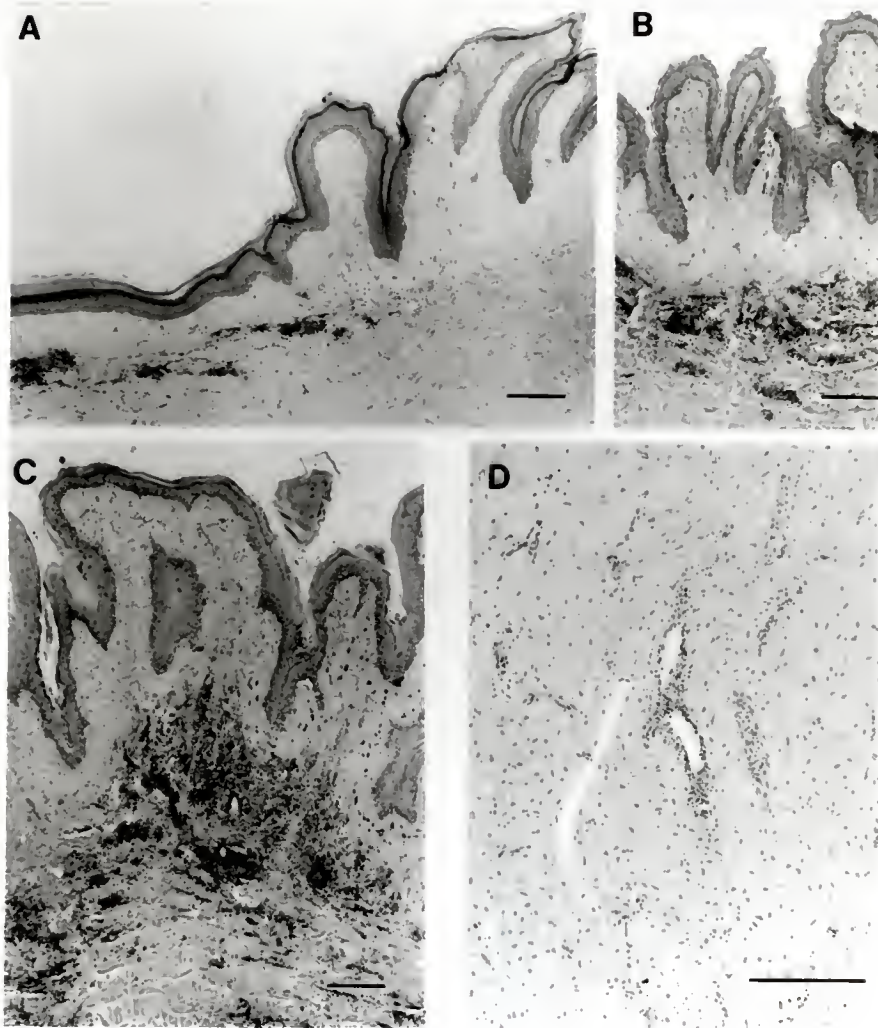


Figure 6-9. Lymphocytic infiltrates observed in green turtle fibropapillomas. (A) Moderate dermal lymphocytic infiltrate at the lateral margin of a fibropapilloma. (B) Extensive lymphocytic accumulation in the reticular dermis along the deep dermal border of a fibropapilloma. (C) Intense lymphocytic infiltration in the deep dermal margins of the tumor with extension into the tumor itself. Within the tumor, this infiltrate would be graded as 3. (D) Mild (grade 2) perivascular lymphocytic infiltrate within tumor stroma. H&E (scale bars = 250 μm).

surrounding connective tissue (Figure 6-9C). Perivasculitis within the fibromatous portion of tumors was seen in about 68.6% of cases. While it was diagnosed more frequently in the Florida samples, the difference was not statistically significant. Perivascular infiltration within the tumors was not associated with severe degenerative changes in the stratum spinosum, ulceration, or the presence of spirorchid ova, but was most strongly associated with cleft formation (Chi-Square = 5.87, $p < 0.025$).

Potential pathogens

Virus-like inclusions. Eosinophilic intranuclear inclusions were observed by H&E in single biopsies from 2 Florida turtles (Figure 6-10), yielding a prevalence estimate of 8% (Table 6-2). Inclusions were found within a focus of early spinous layer degeneration in one case and within a focus of ballooning degeneration adjacent to an ulcer in the second case. Similar inclusions were not observed in Hawaiian samples.

Spirorchid ova. Spirorchid trematode ova (Figure 6-11) were detected more frequently in histologic sections of tumors from Hawaiian turtles (50.9%) than in sections of tumors from Florida turtles (30.3%). These relative frequencies were significantly different (Chi-Square = 6.8, $p < 0.01$). Given the smaller size and number of biopsies per Hawaiian turtle compared to the Florida sample it is apparent that Hawaiian tumors were more heavily infected with

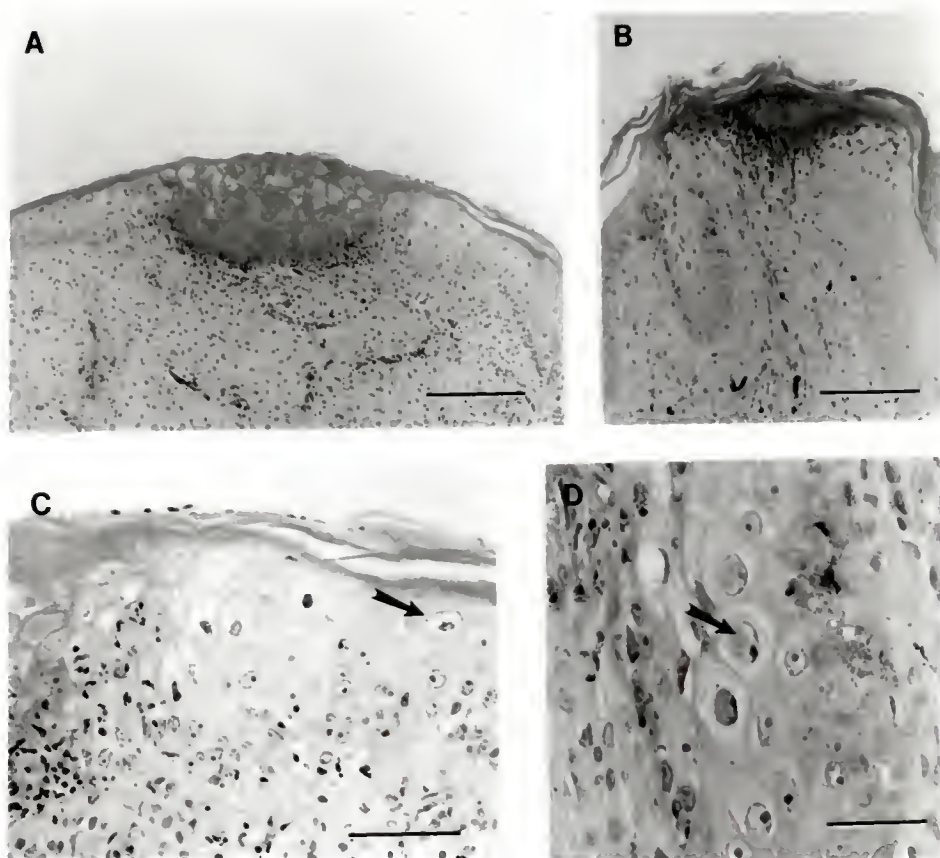


Figure 6-10. Herpesvirus-like intranuclear inclusions. (A) Spontaneous fibropapilloma from a free-ranging Florida green turtle with a focus of epidermal ballooning degeneration at the edge of a cutaneous ulcer (scale bar = 200 μ m). (B) Experimentally-induced fibropapilloma with a similar lesion (scale bar = 200 μ m). (C) Higher magnification of lesion from spontaneous tumor showing eosinophilic intranuclear inclusion (arrow) (scale bar = 100 μ m). (D) Higher magnification of lesion in experimentally induced tumor showing eosinophilic intranuclear inclusion (arrow) (scale bar = 50 μ m). H&E.

Table 6-2. Prevalence estimates of spirochidiasis and herpesvirus infection among turtle population samples

Population	Prevalence (%)			
	N	Herpesvirus		
		Spirochids ^a	Inclusions ^b	Antigens ^c
Hawaii	15	73.3	0	0
Florida	25	52	8	24
Experimental	11	0	36.4	54.5

^a Diagnosed by detection of spirochid ova in tissue sections.

^b Eosinophilic intranuclear inclusions detected in H&E stained tissue sections.

^c Diagnosed by immunohistochemistry.

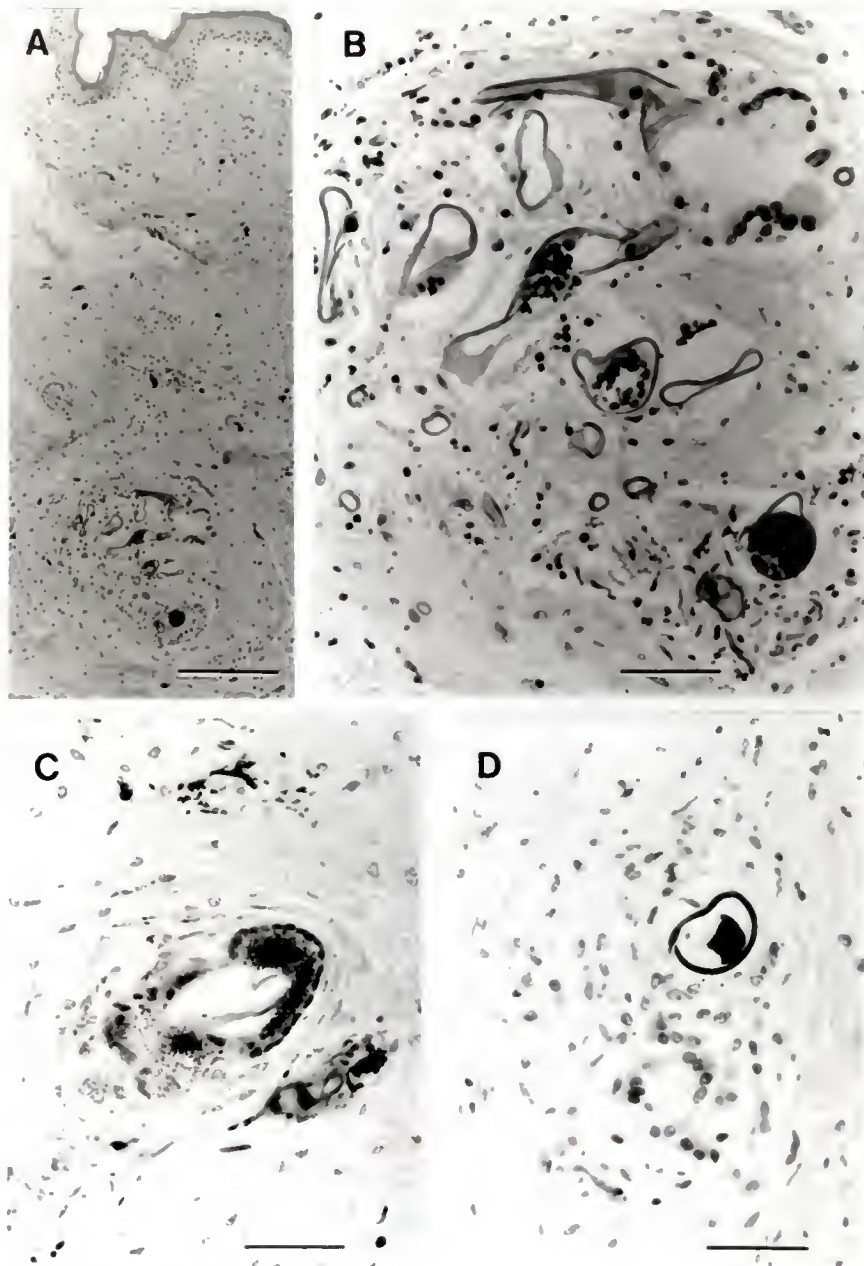


Figure 6-11. Spirochid trematode eggs found in fibropapillomas of free-ranging green turtles. (A) Cluster of ova in the deep dermis (scale bar = 200 μ m). (B) Higher magnification showing multinucleate foreign body cell (scale bar = 50 μ m). (C & D) Examples of single eggs lodged within tumor blood vessels and surrounded by multinucleate foreign body giant cells (scale bars = 50 μ m). Spirochid ova were found both in fibropapillomas and normal tissues of free-ranging green turtles. H&E.

spirorchid eggs than Florida tumors. The prevalence of spirorchidiasis, diagnosed by histopathology, among Hawaiian turtles was 73.3% whereas the prevalence among Florida turtles was only 52% (Table 6-2). However, these relative frequencies were not significantly different.

Bacteria. Bacteria were often found in the desquamating layers of the stratum corneum. Small colonies of cocci as well large bacilli that formed long chains were frequent between epidermal papillae (Figure 6-12A-C). Ulcerated areas were frequently infected. Bacteria were found in a significantly higher number of Hawaiian biopsies (66%) than in Florida biopsies (46.2%) (Chi-Square = 5.8, $p < 0.025$).

Fungi and algae. Classification of eukaryote microbes from H&E stained sections was difficult. Oval basophilic structures 2-3 μm in diameter were widely scattered in the upper stratum corneum. These resembled yeasts but could also represent unicellular algae (Figure 6-12A-C). In one biopsy, a severely necrotic and ulcerated area was infected with invading nonpigmented septate non-branching fungal hyphae (Figure 6-12D). Fungi or algae were found in a higher percentage of Hawaiian samples (32.1%) than Florida samples (6.7%) and this difference was statistically significant (Chi-Square = 18.9, $p < 0.001$).

Metazoan epibionts. Two types of metazoans were occasionally identified. One type, found in 2 Florida biopsies, was attached to smooth epidermis and had a broad

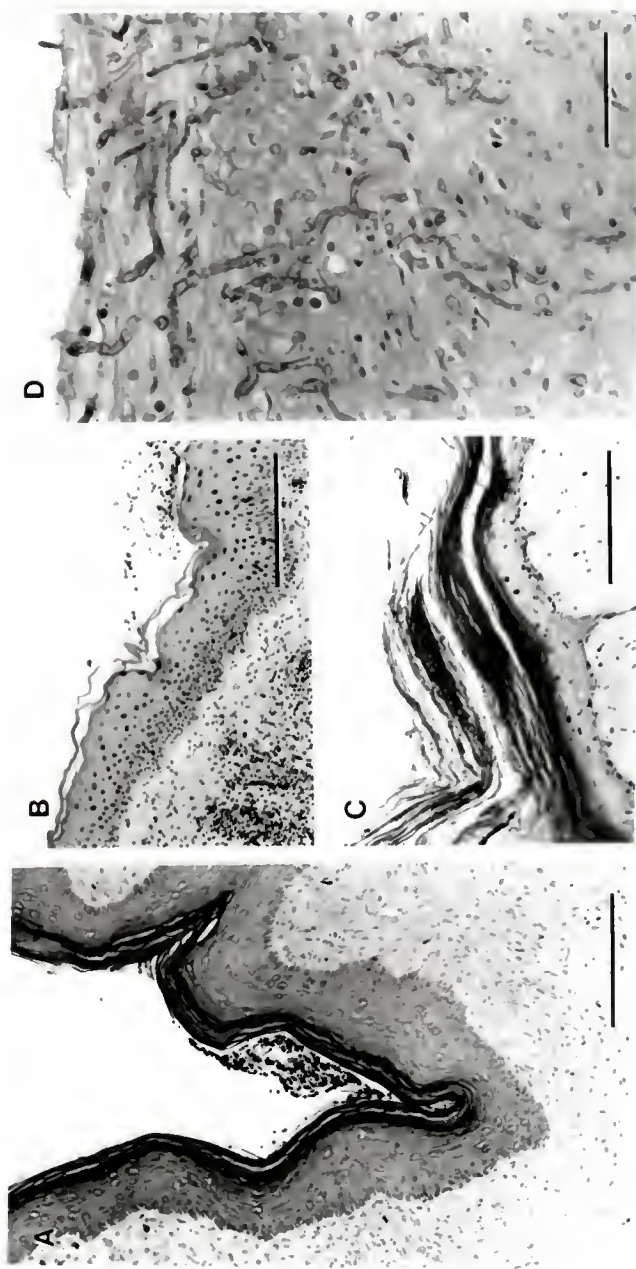


Figure 6-12. Bacteria, fungi, and algae found on cutaneous fibropapillomas (A & B). Clusters of large spherical microorganisms, possibly yeast or algae (scale bars = 200 μm). (C) Bacterial elements within layers of exfoliating cells of the stratum corneum (scale bar = 200 μm). (D) Fibropapilloma showing fungal hyphae within all layers of necrotic epidermis and underlying dermis (scale bar = 50 μm).

base that separated the stratum corneum and reduced the epidermal thickness. This organism was heavily mineralized and thus detail was poor. A thick light pink band separated its base from the epidermis. This organism was interpreted as a barnacle. The second type of metazoan was always located within folds of epidermis (Figure 6-13). In fact there was a statistically significant positive association between the presence of these organisms and having verrucous epidermis at the sub-gross level (Chi-Square = 4.65, $p < 0.05$). These organisms had multiple legs and gills and other structures suggesting that they were arthropods, possibly crustaceans or mites. Even though the Florida biopsy collection contained more grossly verrucous tumors, the Hawaiian biopsies had a higher frequency of metazoans than the Florida sample (18.9% versus 7.6%) and this difference was statistically significant (Chi-Square = 4.65, $p < 0.05$).

Experimentally Induced Cutaneous Tumors

Tumor biopsies were collected from 11 turtles with experimentally induced GTFP from the 1993 transmission study (Chapter 4). Thirty-eight tumors were biopsied (2-7 tumors per turtle, $\bar{x} = 3.5 \pm 1.6$). The biopsies were taken between 1 and 28 weeks after each tumor was first detected, and between 37 and 56 weeks after turtles were experimentally inoculated. The histologic features of these biopsies were described using the same criteria used for spontaneous tumors.

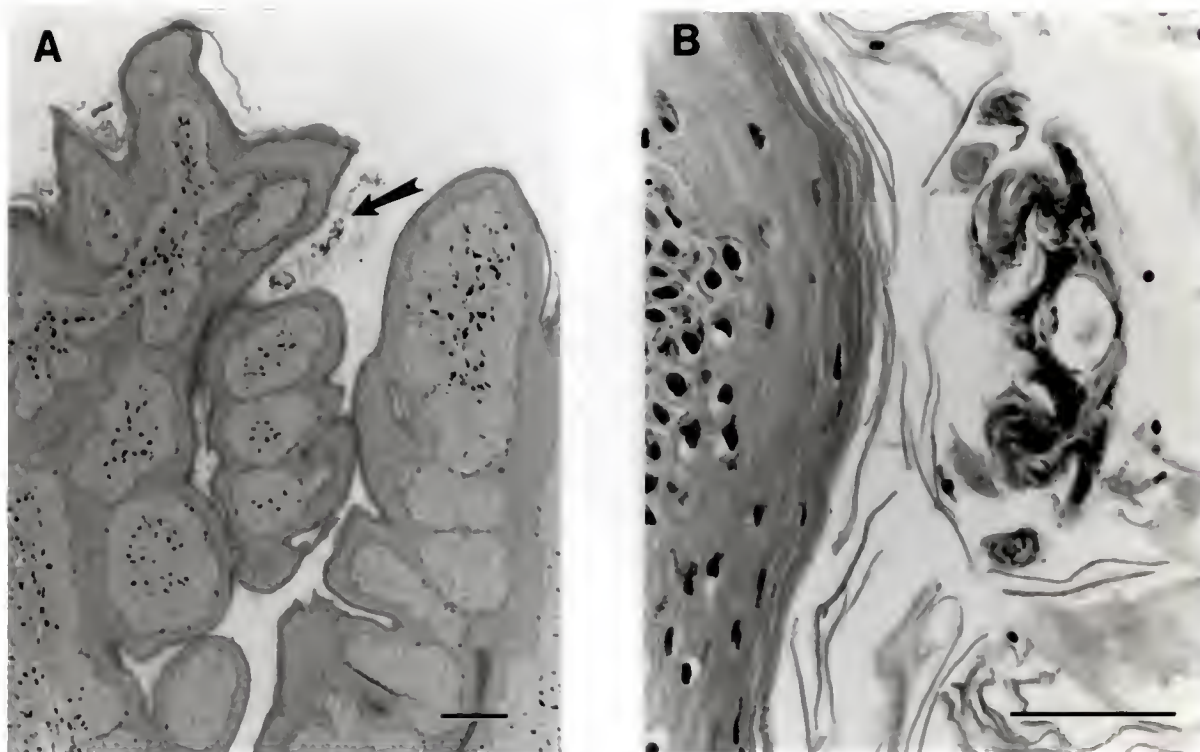


Figure 6-13. Metazoan epibionts. (A) Arthropods (arrow) were found occasionally within epidermal folds of fibropapillomas (scale bar = 200 μ m). (B) Higher power showing some anatomic detail (scale bar = 50 μ m). H&E.

Differences between spontaneous and experimental tumors

Table 6-3 compares the relative frequencies of various histologic features in experimentally induced tumors with those in spontaneous tumors of free-ranging Hawaiian and Florida turtles. Statistically significant differences between groups in relative frequency of most features were not found. Those features that were significantly different are presented below.

Pigmentation. There were relatively more pigmented tumors in the experimental group (73.7%) compared with the spontaneous group (54.7%) and this was significantly different (Chi-Square = 4.6, $p < 0.05$).

Degenerative changes in the spinous layer. A higher percentage of experimentally induced tumors had evidence of degeneration in the stratum spinosum (71.1%) compared to spontaneous tumors (22.1%) and this difference was statistically significant (Chi-Square = 34.7, $p < 0.001$).

Lymphocytic infiltrates. Although there was no difference between spontaneous and experimentally induced tumors in the percentage of sections with clefts, relatively fewer experimentally induced tumors had detectable perivascular lymphocytic infiltrates (36.8%) than spontaneous tumors (68.6%) and this difference was statistically significant (Chi-Square = 13.5, $p < 0.001$).

Virus-like inclusions. Eosinophilic intranuclear inclusions (Figure 6-10B,D) were detected in 5 (13.2%) of the

Table 6-3. Comparison of the gross and histologic features of spontaneous and experimentally induced fibropapillomas

Observations	Relative Frequency (%)		Chi-Square
	Spontaneous (N = 172)	Induced (N = 38)	
Gross Examination			
Pigmentation	54.7	73.7	p < 0.05
Epidermal Folding	68.1	73.7	ns
Histopathology			
Acanthosis	94.2	100	ns
Orthokeratosis	81.4	94.5	ns
Basal Cell Degeneration	86.6	81.6	ns
Dermal-Epidermal Cleft	53.5	50.0	ns
Spinous Layer Degeneration	22.1	71.1	p < 0.001
Intraepidermal Pustule	18.0	28.9	ns
Ulceration/Erosion	37.8	36.8	ns
Inflammation			
Granulocytes	43.0	50	ns
Foreign Body Granuloma	43.6	0	p < 0.001
Perivascular Lymphocytes	68.6	36.8	p < 0.001
Infiltrating Lymphocytes	-	76.3	-

Table 6-3--continued.

Observations	Relative Frequency (%)		Chi-Square
	Spontaneous (N = 172)	Induced (N = 38)	
Potential Pathogens			
Virus-like Inclusions	1.2	13.2	p < 0.001
Bacteria/Fungi	54.1	63.2	ns
Metazoan Epibionts	12.2	2.6	ns
Spirorichid Ova	36.6	0	p < 0.001

experimentally induced tumors by examination of H&E stained sections, compared to only 2 (1.2%) in the spontaneous tumors. This difference was statistically significant (Chi-Square = 13.8, $p < 0.001$).

Spirorchid ova. Neither spirorchid ova nor foreign body granulomas were found in any experimental tumors. The differences in relative frequencies of ova and associated foreign body granulomas between experimental and spontaneous tumors were statistically significant (Chi-Square = 19.9, $p < 0.001$ and Chi-Square = 25.9, $p < 0.001$ respectively).

Early pathologic changes in experimentally induced tumors

Five experimentally induced tumors were biopsied when they were about 1-2 weeks old. These were raised plaques or sessile masses measuring < 5 mm in diameter. The earliest changes identified in these biopsies were proliferation of fibroblasts in the papillary layer of the dermis and proliferation in the stratum germinativum (Figure 6-14). Superficially, the proliferating dermis resembled granulation tissue with linear blood vessels aligned perpendicular to the skin surface. Even in the earliest clinically detectable tumors, however, some areas had papillary projections (epidermal folds) and evidence of orthokeratosis. In addition, sporadic basal cell degeneration was also noted and in 3 cases clefts had already formed at the dermal-epidermal junction. In 3 cases degeneration in the stratum spinosum was noted that progressed to intraepidermal pustule formation in

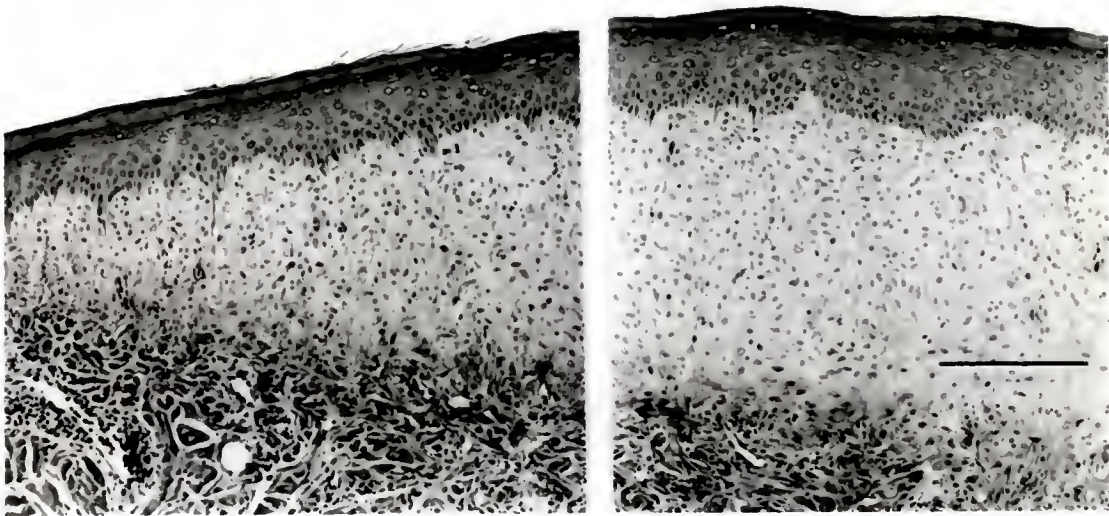


Figure 6-14. Early pathologic changes in experimentally induced fibropapillomas. This tumor was biopsied approximately 1 week following detection and shows proliferation in the papillary dermis overlaid by moderately hyperplastic epidermis. New blood vessels are aligned perpendicular to the skin surface in a manner resembling granulation tissue. H&E (scale bar = 200 μ m).

one case and ulceration in another. Although lymphocyte aggregates were noted in the deep dermal margins of 3 early tumors, lymphocytic infiltration into the tumor stroma itself was noted only in one case.

In older (> 3 weeks) and larger experimentally induced tumors, all the proliferative and degenerative changes were found, with no obvious correlation between various features and tumor age or size.

Herpesvirus Immunohistochemistry

An antiserum showing specific immunoreactivity with herpesvirus intranuclear inclusions was used to screen tumor sections for evidence of productive herpesvirus infection. Positive intranuclear immunoreactivity was detected focally in the spinous layer, only in keratinocytes undergoing early to advanced ballooning degeneration (see Figure 6-1). Immunohistochemistry was more successful than H&E at identifying herpesvirus-associated cytopathology. This was probably because the antiserum was reactive with herpesvirus antigens expressed early in productive infection whereas the formation eosinophilic inclusions is a relatively late event (Gelb, 1993a; Knipe, 1990).

Spontaneous tumors. Nine of the 119 spontaneous tumors (7.5%) from Florida green turtles had herpesvirus inclusions detected by immunohistochemistry. These herpesvirus-positive biopsies were from 6 turtles, yielding a 24% prevalence estimate for Florida green turtles (Table 6-2). In contrast,

none of the 53 biopsies from 15 Hawaiian turtles were diagnosed with herpesvirus by immunohistochemistry.

Experimentally induced tumors. The tissue blocks from 4 of the 5 biopsies originally diagnosed with herpesvirus inclusions by H&E were destroyed during processing for electron microscopy and were not available for further testing (Chapter 4). The remaining block was found to be positive by immunohistochemistry. In addition, positive immunohistochemical reactions were detected in 4 new tumors. Thus, evidence of herpesvirus infection was detected in 9 (23.7%) of the biopsies from 6 experimental turtles, yielding a prevalence of 54.5% infected (Table 6-2).

Immunofluorescence for Antibody Deposition

The clefts that developed at the junction of dermis and epidermis in approximately half of spontaneous and experimental tumors were suggestive of an immune-mediated process. Immunofluorescence labelling of frozen sections, from 2 biopsies containing clefts, with monoclonal antibodies to green turtle IgM, 7S IgY, or 5.7S IgY, yielded negative results. Thus, antibody deposition in the stratum basale, basement membrane, or superficial dermis was not detected in tumors undergoing cleft formation.

Visceral Tumors

Twenty-two visceral tumors were examined from 9 Florida turtles. These included samples from 11 lung, 6 kidney, 2 heart, 1 liver, 1 mesenteric, and 1 stomach mass. A sample of

a small intestinal mass from 1 Hawaiian turtle was not diagnostic. Visceral tumors were mostly smooth, firm, and white but some were gelatinous and translucent (see Chapter 2, Figure 2-3). Tumors were embedded within normal tissue parenchyma and bulged from the surface. Most visceral tumors appeared to be well demarcated from surrounding tissue but some kidney tumors had irregular borders suggesting infiltration of surrounding stroma.

The common histologic feature of visceral tumors was extensive fibroblast proliferation leading to myxoma or fibroma formation (Figure 6-15). Fibroblast proliferation with abundant secretion of mucinous (myxomatous) ground substance appeared to be an early development, followed by increased collagen deposition in more mature tumors (Figure 6-15A). In the lungs, fibrosis occurred in the bronchial interstitium and as the mass expanded, islands of normal bronchial epithelium became entrapped within the fibroma (Figure 6-15B). Similarly, in the kidney, masses began as extensive interstitial fibrosis that eventually separated and entrapped renal tubules (Figure 6-15C). Similar patterns of interstitial fibroplasia were seen in liver (Figure 6-15D), stomach, and heart. Epithelial components involved in visceral nodules were histologically normal except for occasional foci of bronchial epithelial hyperplasia in some lung tumors. Occasional separations between bronchial epithelium and underlying fibroma were seen and could be

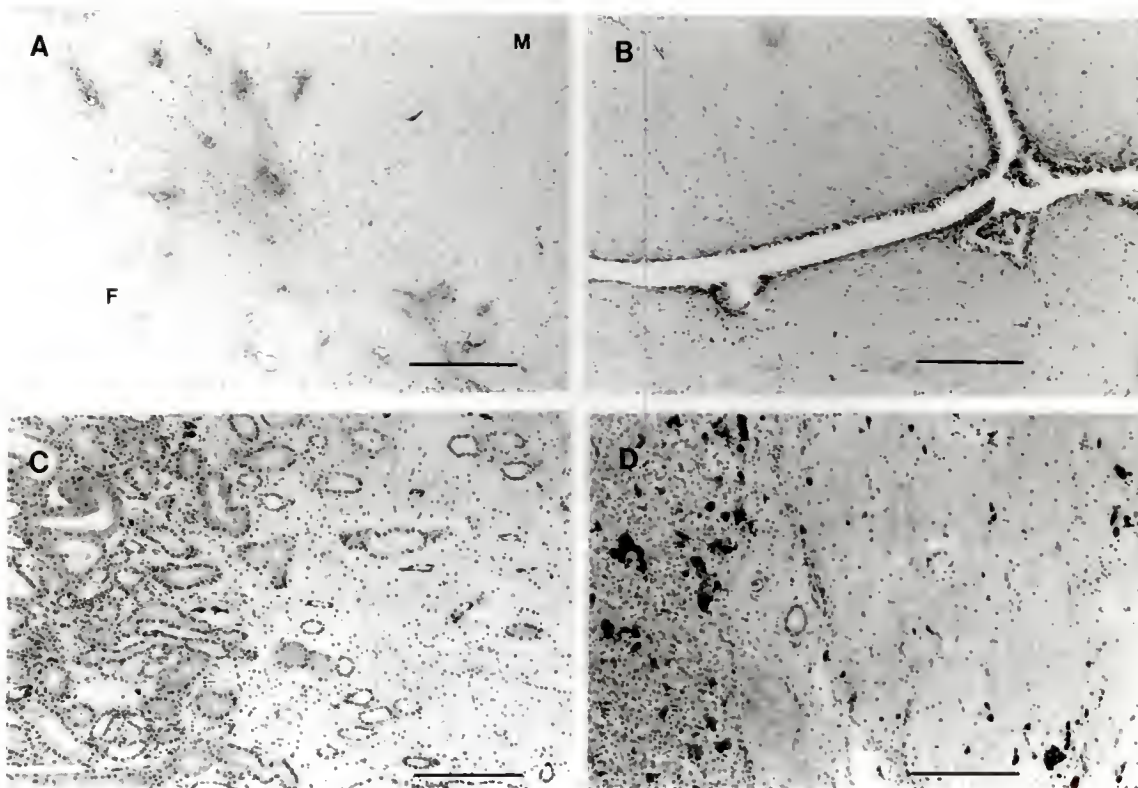


Figure 6-15. Histologic features of visceral tumors found in some green turtles with cutaneous GTFP. (A) Lung nodule showing the transition from collagenous extracellular matrix in a fibrous nodule (F) to a more myxomatous extracellular matrix in an adjacent myxoma (M). (B) Lung nodule showing extensive fibrous proliferation entrapping relatively normal bronchial epithelium. (C) Kidney tumor showing transition from normal renal parenchyma to tumor tissue. Extensive interstitial fibrosis leads to entrapment of normal renal tubules from normal parenchyma to tumor. H&E (scale bars = 200 μ m).

attributed to edematous changes below the basement membrane. Trematode ova with associated foreign body granulomas were found both in visceral fibromas and in adjacent normal tissue.

Discussion

Smith and Coates (1938) provided the first histologic descriptions of GTFP. Subsequently, Jacobson et al. (1989) provided a more detailed description of light and electron microscopic features of GTFP and these basic histological findings have been confirmed by others (Aguirre et al., 1994b; Brooks et al., 1994; Harshbarger, 1991; Jacobson et al., 1991; Norton et al., 1990; Williams et al., 1994). Similarly, all of the histologic features of spontaneous tumors that were described in this study have been noted to various degrees in earlier reports (see Chapter 2). This study, however, attempted to provide a plausible scenario for the pathogenesis of GTFP based upon an analysis of the histopathologic evidence.

Florida Versus Hawaiian GTFP

In general, cutaneous GTFP biopsies from Hawaiian turtles and those from Florida turtles had similar histologic characteristics. Except for eosinophilic intranuclear inclusions, which were not found in Hawaiian biopsies, all described histologic features were found in both population samples to varying degrees.

Significantly fewer Florida biopsies had surface bacteria, fungi, or metazoans compared to Hawaiian samples. Although these differences in epibiota may be real, this result may have been an artifact of the way in which turtles were handled. Most of the Hawaiian samples were collected in the field from free-ranging turtles that were captured, biopsied, and then released. Most of the Florida samples came from stranded turtles that were brought to rehabilitation facilities for treatment. These turtles were often washed and tumors scrubbed prior to surgical excision. Thus, it is not surprising that surface contaminants and epibionts were found less frequently in the Florida samples.

Spirorchidiasis was diagnosed more frequently in Hawaiian samples than in Florida samples. The fact that there were fewer biopsies taken per Hawaiian turtle, on average, and that these biopsies were smaller than Florida ones, indicates that Hawaiian tumors had a heavier spirorchid ova burden than Florida tumors. This difference may reflect higher fecundity among Hawaiian spirorchids, higher adult fluke burdens, or more persistent infections in Hawaiian green turtles.

The higher percentage of tumors from Florida with basal cell degeneration may indicate that more Hawaiian biopsies were from older tumors in which epidermal pathology was no longer active, i.e. with healed epidermis. Alternatively, this difference may be a sampling artifact. Because the

Hawaiian biopsies were smaller (6 mm punch biopsies) than the Florida samples (excisional biopsies), less tumor surface area was represented, and combined with the sporadic distribution of early basal layer changes, it is not unexpected to have this lesion under-represented in Hawaiian samples. The same sampling artifact may explain why herpesvirus-like inclusions were not detected by H&E or immunohistochemistry in any of the Hawaiian samples.

Spontaneous Versus Experimentally Induced GTFP

Experimentally induced tumors had the same group of proliferative and degenerative features as seen in spontaneous GTFP. However, experimentally induced tumors had no evidence of spirorchidiasis or of the foreign body granulomas associated with spirorchid ova. The absence of spirorchids and the rarity of metazoan epibionts in experimental tumor biopsies can be explained by the fact that experimental turtles were housed in tanks supplied with sand-filtered seawater (Chapter 4). As discussed previously (in Chapter 4), the absence of spirorchid ova from these tumors eliminates any direct role for these organisms in GTFP pathogenesis. Also, the close association of foreign body granulomas with spirorchid ova indicates that this inflammatory process is an incidental finding in GTFP.

Compared to spontaneous tumors, the experimental tumors were significantly more likely to have severe degenerative changes in the stratum spinosum and less likely to have

lymphocytic perivascular infiltrates within the tumor stroma, although lymphocytic infiltrates were commonly observed at the junction of tumor and normal stroma. These differences may be due to differences in tumor age and progression or variation in the duration of host immune responses to the GTFP agent. Experimental turtles had clinically apparent tumors for no longer than 7 months when biopsies were taken. In contrast, the age of individual spontaneous tumors or the duration that free-ranging turtles had GTFP were unknown. Thus, experimental turtles were more likely to have early active infections whereas free-ranging turtles probably had more chronic disease.

These age/stage differences in tumor development and host immune response may also explain why eosinophilic intranuclear inclusions and herpesvirus antigens were found more commonly in experimentally induced tumors.

Pathogenesis of Tumor Progression

It is difficult to identify stages of progression from a collection of tumor biopsies taken from free-ranging turtles because the duration of exposure to the GTFP agent, the age of individual tumors, and the immunological history of the turtles are unknown. Experimentally induced tumors provide a resource for describing tumor progression because the exposure history of the patient and the age of individual tumors are known.

The following description of tumor progression is a hypothesis, proposed to account for all of the pathologic changes observed in GTFP, assuming that a single agent, the GTFP-associated herpesvirus, is responsible. However, until tumors can be produced experimentally with a single proven etiologic agent, it will remain unclear whether all pathologic features discussed below can be attributed to the GTFP agent or if some result from secondary processes.

Invasion and early infection. Presumably, the agent invades skin, either directly or through minor wounds, and infects dermal fibroblasts and basal epidermal cells. Infection triggers uncontrolled proliferation of infected dermal fibroblasts which continues to various degrees throughout the ontogeny of the tumor. Infected basal cells are also stimulated to proliferate and differentiate, resulting in various degrees of acanthosis and, in cornified epithelium, orthokeratotic hyperkeratosis. This epidermal hyperplasia may in part be a response to paracrine stimulation from infected fibroblasts and vice versa (Michalopoulos, 1989). However, it is logical to assume that both epidermis and dermis are infected because virus is produced in the epidermis and visceral fibromas with normal epithelial components can form (see below). Infection of basal epidermal cells can lead to rapid proliferation and differentiation or degeneration, depending on the ability of the cell's homeostatic mechanisms to compensate for

infection, and possibly on the influence of anti-viral host defenses e.g. interferon (Lebel & Hirsch, 1985; Pestka et al., 1987).

Productive infection. Productive viral infection occurs in keratinocytes of the stratum spinosum, the onset of which causes ballooning and reticular degeneration and intraepidermal vesicle formation. When vegetative virus production is completed before cells die and lyse, these swollen cells will contain eosinophilic intranuclear inclusions. However, if the cells degenerate and die early, due to loss of homeostatic compensation or to host anti-viral responses, these necrotic cells will not contain intranuclear inclusions. Granulocytes are recruited to intact or ulcerated vesicles, the former resulting in intraepidermal pustules. Cleft formation secondary to basal cell degeneration or host immune responses to infected epithelium also contribute to cutaneous ulceration.

Resolution. Following active infection, the epidermis begins to heal over the fibromatous portion of the tumor. The healing epidermis is hyperplastic, resembling proliferating early-infected epidermis, but without the degenerative changes in basal or spinous layers. The dermal portion of the tumor may continue to proliferate since it contains non-productively infected (transformed) fibroblasts. On the other hand, the fibroma may regress as non-productively infected cells are destroyed or arrested by the host's cell-mediated

immune responses, or as environmental conditions become sub-optimal for further proliferation. While it is clear from field studies that fibropapillomas can regress (see Chapter 2), the mechanism of tumor regression is not clear. There is ample evidence among poikilotherms of seasonal and temperature effects on tumor progression and regression (Asashima et al., 1982; Bowser & Wooster, 1991; Sonstegard, 1976). Cell-mediated immunity is probably important (Aiba et al., 1986; Allison, 1967; Tagami et al., 1974) and it is possible that regression is mediated by direct cytotoxicity or anti-proliferative effects of cytokines produced by lymphocytic infiltrates (Okabayashi et al., 1993). Little evidence of direct tumor destruction by infiltrating lymphocytes was found in the series of biopsies examined in this study.

Pathogenesis of Dermal-Epidermal Clefts

Dermal-epidermal separation (cleft formation) was observed to occur independently of primary degenerative changes observed in the stratum spinosum and is interpreted as a distinct process. Cleft formation seemed to result from several possibly related processes even within the same biopsy including separation of basement membrane from edematous papillary dermis (subepidermal cleft), basal cell degeneration (intraepidermal cleft), and lymphocytic infiltration at the dermal-epidermal junction (both). These multiple pathologic features are similar in characteristics

to erythema multiforme in humans, which often occurs as a sequela to herpesvirus infection (Howland et al., 1984; Lever & Schaumburg-Lever, 1983; Shelley, 1967). This explanation would be consistent with a proposed herpesvirus etiology for GTFP. However, spongiosis and basal cell necrosis with separation between basal cells and suprabasal cells has also been observed in regressing bovine papillomavirus-induced fibropapillomas (Lee & Olson, 1968) and in regressing human warts (Tagami et al., 1974). In addition, other conditions that are characterized by epidermal-dermal cleft formation must be considered. Among these are: the autoimmune diseases such as bullous pemphigoid, and lupus erythematosus, in which auto-antibodies are directed against basement membrane or basal cells, and mechanobullous diseases such as epidermolysis bullosa, in which defects in basement membrane attachment to the dermis lead to cleft formation (Lever & Schaumburg-Lever, 1983).

Direct immunofluorescence staining with monoclonal antibodies to green turtle immunoglobulin classes did not detect antibody deposits within clefts or within adjacent tissue. Because false-negative staining of clefts may occur in some immune-mediated diseases (Soltani, 1986), these result cannot disprove the involvement of autoimmune antibodies, although they suggest that autoimmunity is not a major feature in the pathogenesis of these clefts. Tests for other components possibly involved in immune mediated cleft

formation, such as complement deposition could not be conducted.

Possible Routes of Dissemination

The fact that turtles develop multiple cutaneous tumors, with an often bilaterally symmetrical distribution, as well as visceral fibromas suggests that the infectious agent is disseminated systemically. Dissemination to multiple cutaneous sites can occur via the vascular system or transport along peripheral nerves. Both of these routes of dissemination are characteristic of herpesvirus infections (Gelb, 1993b) and would be consistent with a herpesvirus etiology for GTFP.

Visceral tumors could develop as metastases of transformed fibroblasts from the primary tumor(s) or as a result of multicentric infection of fibroblasts during systemic dissemination. The latter possibility is more likely because, GTFP fibroblasts do not have any other features suggestive of malignancy (Papadi et al., 1995; see Chapter 8). If multiple infection is involved then it is probable that visceral organs lack a permissive epithelium for vegetative virus replication because the epithelial components found in visceral fibromas are normal.

Can Herpesvirus Explain GTFP Pathology?

The proposed scenario for the pathogenesis GTFP assumes that all of the observed histopathologic changes are caused by a single etiologic agent. The following discussion will

focus on whether it is reasonable to assume that a herpesvirus fulfills this role.

All of the histologic features occurring in the epidermis can be explained by herpesvirus. Acanthosis, degeneration, and necrosis in the stratum germinativum (stratum basale and stratum spinosum), followed by ulceration are hallmarks of herpesvirus dermatitis (Cheville, 1988; Gelb, 1993b; Lever & Schaumburg-Lever, 1983) and have been demonstrated in grey patch disease of green turtles (Rebell et al., 1975). In addition, although non-specific, the perivascularitis and pathologic changes occurring along the epidermal-dermal junction are consistent with post-herpetic erythema multiforme. The fact that herpesvirus-like intranuclear inclusions and herpesvirus antigens were detected in association with epidermal pathology in both spontaneous and experimentally induced tumors is strongly suggestive of a herpesvirus etiology.

On the other hand, herpesviruses are not known to cause fibropapillomas or fibromas in vertebrates, although some may transform fibroblasts in vitro (Tevethia, 1985) and fibromas associated with herpesvirus infection have been described in african elephants (Jacobson et al, 1986b). In contrast, poxviruses, retroviruses, and papillomaviruses have been shown to cause spontaneous fibromas and sarcomas in a variety of vertebrate species (Chapter 2). Of these alternate agents, it is most important to rule out papillomaviruses because

they are known to cause fibropapillomas in several mammalian species (Sundberg, 1987).

Can Papillomavirus Explain GTFP Pathology?

So far there is no electron microscopic or molecular biological evidence for productive papillomavirus infection in GTFP (Chapter 2). In addition, papillomaviruses are not known to cause degenerative changes in the stratum germinativum like those observed in GTFP. Productive papillomavirus infection usually causes hyperkeratosis, often with retention of nuclei containing basophilic intranuclear inclusions in the stratum corneum (parakeratosis) (Cheville, 1988; Lever & Schaumburg-Lever, 1983). Infected cells in the upper epidermis often develop intracellular edema resulting in a perinuclear halo of cleared cytoplasm (koilocytosis) (Cheville, 1988). Although intracellular edema leading to perinuclear halo formation was seen in many tumors, parakeratosis was not a feature of GTFP. In addition, electron microscopic examination of superficial keratinocytes in foci of koilocytosis were negative for papillomavirus particles.

The possibility of a dual infection, however, with a primary papillomavirus etiology for fibroepithelial proliferation and a secondary herpesvirus etiology for epidermal pathology, cannot be ruled out by the histopathologic findings of this study. However, the chloroform sensitivity of the GTFP agent (Chapter 5) suggests

that an enveloped virus is the etiologic agent. It also remains possible that another enveloped virus, such as a retrovirus, induces the fibroepithelial proliferation characteristic of GTFP and that the proliferating epidermis provides a favorable environment for the recrudescence of a latent herpesviral infection.

To prove that the GTFP-associated herpesvirus is the cause of GTFP, this virus must be shown to induce fibroblast proliferation in green turtles. Experimental induction of fibropapillomas with purified herpesvirus or demonstration of herpesvirus transforming genes in GTFP derived fibroblasts would provide the evidence needed to support a herpesvirus etiology.

CHAPTER 7
SEROLOGIC EVIDENCE FOR THE ASSOCIATION BETWEEN
SPIRORCHIDIASIS, HERPESVIRUS INFECTION, AND
FIBROPAPILLOMATOSIS

Introduction

Serology can provide indirect evidence to support a particular hypothesis about the etiology of an infectious disease. Turtles that have developed clinical fibropapillomatosis should have antibody directed against the etiologic agent. Previously naive turtles that develop experimentally induced tumors should convert to seropositivity for the GTFP agent. Seropositivity to incidental pathogens should have no association with tumor status.

Two pathogens, herpesvirus and spirorchid ova, have been found within fibropapillomas and each has been hypothesized to cause GTFP (see Chapter 2). Transmission experiments (Chapter 4) show that GTFP is caused by a filterable agent and histopathological and immunohistochemical analyses (Chapter 6) tend to support the hypothesis of a herpesvirus etiology. The GTFP-associated herpesvirus was first reported in 2 green turtles with spontaneous GTFP (Jacobson et al., 1991). Herpesvirus inclusions have since been found in

additional cases of spontaneous disease and in experimentally induced tumors (Chapters 4 & 6).

Although spirorchid ova have been ruled out by experimental transmission studies as a direct cause of GTFP, an indirect role for spirorchidiasis in the epizootiology of GTFP remains possible. For example, migrating blood flukes may facilitate infection with the GTFP agent by damaging tissues, especially skin. Cercariae may serve as vectors of the GTFP agent in endemic areas. Trematodes may facilitate the development of GTFP by severely debilitating their host (Glazebrook et al., 1981; Glazebrook & Campbell, 1990a, 1990b; Wolke et al., 1982), so that the immune system cannot eliminate the GTFP agent before tumors develop. Spirorchid egg deposition within tumor vasculature may trigger a chronic inflammatory and immune response that could result in either a continuing hyperplastic response within tumors or eventual tumor rejection. The effect of concurrent parasitic diseases as a cofactor in the pathogenesis of GTFP deserves further investigation.

This chapter describes the development of crude diagnostic tests to test the strength of association between antibodies against spirorchids or herpesvirus and GTFP status. This study provides further indirect evidence supporting the hypothesis that GTFP is caused by herpesvirus.

Materials and Methods

Plasma Samples

Free-ranging turtles. Plasma samples were collected from free-ranging green turtles netted at two study sites on the Atlantic coast of Florida. These two sites have been monitored since 1982 and 1988 by Llewellyn Ehrhart (University of Central Florida, Orlando, FL 32816), and represent two distinct near-shore feeding habitats for juvenile green turtles. These sites are in close geographic proximity and have markedly different GTFP prevalences. The Indian River Lagoon site (Indian River Co., FL) is located lat. 27°49'57 N, long. 80°26'18 W and consists of a shallow (1-3 m), muddy bottom with drift algae and seagrass beds. GTFP prevalence at this site has averaged 50% since monitoring began in 1982 (Ehrhart, 1991; Ehrhart, pers. comm.). The Wabasso Beach site is located lat. 27°47'38 N, long. 80°24'34 W, approximately 1 km due east of the lagoon site and is a Sebellariid worm reef located on sandy bottom in about 2-3 m of water. The primary forage at this site is algae. GTFP prevalence at this site has been 0% since monitoring began in 1988 (Ehrhart, 1991, pers. comm.).

Transmission study turtles. Plasma was collected from captive-reared turtles that had been raised from eggs in filtered sea water and used in various transmission studies. This series included plasma samples collected prior to inoculation from each of 20 turtles used in the 1993

transmission study (Chapter 4) and matched plasma samples for the 19 survivors, collected approximately 1 year post-inoculation. In addition, plasma from the 4 donor turtles used in the 1993 transmission study were collected for testing. Blood samples (3-10 ml) were collected in heparinized syringes from the dorsal cervical sinus (Owens & Ruiz, 1980). Plasma was separated by centrifugation and frozen in aliquots at -20°C and then stored at -70°C.

Detection of Antibodies to the GTFP-Associated Herpesvirus

Plasma antibody reactivity to herpesvirus inclusions was detected using an immunohistochemical assay. Herpesvirus inclusion positive tissue sections were identified by immunohistochemical screening using a known positive plasma, obtained from a 1991 transmission study turtle that developed disseminated cutaneous GTFP (Chapter 6). Once identified, at least twenty 6 µm sections were cut from each paraffin embedded tissue block, mounted on silanized glass slides, and used as antigen substrates for screening unknown plasma samples.

Substrate tissue sections were deparaffinized in 3 changes of xylene and rehydrated through a graded alcohol series. Endogenous peroxidase activity was quenched by incubating slides in 3% H₂O₂ for 10 minutes. Antigenicity was recovered by incubation in an enzyme solution (0.125% trypsin, 0.1% CaCl₂ in PBS; pH 7.4) for 20 minutes at 37°C.

Slides were then washed for 30 minutes in three changes of PBS and blotted dry. Slides were then flooded with test plasma diluted 1:10 in PBS and incubated overnight at room temperature in a humidified chamber. Positive control plasma (diluted 1:50) and negative control plasma (diluted 1:10), obtained from a healthy captive-reared turtle, were run with test plasma. Following incubation with antisera, the sections were washed for 30 minutes in 3 changes of PBS, blotted, and incubated with biotinylated secondary antibody solution for 1 hour. The secondary antibody was a cocktail of biotinylated monoclonal antibodies HL673 (1 $\mu\text{g/ml}$) and HL857 (1 $\mu\text{g/ml}$), specific for green turtle immunoglobulin light chain and 7S IgY heavy chain, respectively (see Chapter 3), in PBS containing normal mouse serum (1:20 dilution). The sections were washed for 30 minutes in 3 changes of PBS, blotted, and incubated for 1 hour with horseradish peroxidase conjugated strepavidin (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:300 in PBS. After a final wash procedure the slides were immersed in substrate (3,3 diaminobenzidine 500 $\mu\text{g/ml}$ in ice cold PBS) and incubated for 2-10 minutes. The color reaction was monitored in control slides. Sections were counter-stained with Harris's hematoxylin, dehydrated, and mounted. Plasma showing specific intranuclear immunoreactivity within foci of ballooning degeneration were considered positive.

Detection of Antibody Reactivity to Spirorchid Trematodes

Diagnosis of spirorchidiasis. Post mortem examinations were conducted on stranded or injured green turtles that were euthanized or died spontaneously. The heart and major blood vessels were opened and examined for cardiovascular flukes. Blood was rinsed through a fine sieve (50 mesh standard) to recover flukes. The gastrointestinal tract and urogenital tract and their contents were examined for helminths. Adult trematodes were collected, identified, and sorted by species by Dr. Ellis Greiner, University of Florida, Gainesville, FL 32610). Voucher specimens were fixed, cleared, and mounted. Except for a small specimen taken for histopathology, the spleen was digested in trypsin (0.25% in PBS) for 8-12 hours at 37°C. The resulting digest was then centrifuged for 10 minutes at 500 x g and the pellet resuspended in PBS. This material was layered onto a Percoll (Pharmacia LKB, Uppsala, Sweden) step gradient (80% Percoll stock in PBS) and centrifuged for 30 minutes at 500 x g to separate trematode ova from tissue debris. Similar digests of fibropapillomas and fibromas collected from those turtles with GTFP were prepared by Dr.E. Greiner. Tissue specimens were fixed in 10% buffered formalin, processed routinely, and 6 µm sections were stained with H&E and examined for spirorchid ova by light microscopy.

Antigen preparation. Two species of adult spirorchids trematodes were found at necropsy in 3 turtles. Forty adult

Learedius learedii were homogenized in PBS/az using a Tekmar Tissuemizer (Tekmar, Cincinnati, OH, USA) and followed by sonication. The resulting extract was filtered through 0.2 μm filter adjusted to a protein concentration of approximately 625 $\mu\text{g/ml}$ and frozen in aliquots at -70°C . Antigen from 3 adult Haplotrema sp. was prepared in the same way.

Enzyme-Linked Immunosorbent Assay. An enzyme linked immunosorbent assay (ELISA) to detect turtle 7S IgY antibodies against Learedius or Haplotrema adult antigens was developed using biotinylated monoclonal antibody (HL857), which has proven specificity for the green turtle 7S IgY heavy chain (Chapter 3). Optimization experiments were performed using positive and negative control plasma obtained from 2 adult spirorchid positive turtles and 3 spirorchid negative turtles, respectively, as determined by necropsy, spleen digest, and histopathology. Learedius learedii crude antigen stock was diluted to concentrations ranging from 0.5-20 $\mu\text{g/ml}$ in PBS/az and 50 μl of each concentration was applied to 12 wells of an ELISA plate (Maxisorp F96, NUNC, Kamstrup, Denmark). Plates were allowed to incubate overnight at 4°C . The ELISA plates were then washed 4 times in an automated plate washer with PBS-Tween. Plates were then blocked for 1 hr at room temperature with 1% BSA in PBS. After washing 4 times with PBS-Tween, each well with a given antigen coating concentration received positive control plasma diluted from 1:10 through 1:640 in PBS, to form a

checker board pattern of antigen and plasma dilution combinations. Negative control plasma was also tested, and 12 wells were used as blank (PBS) controls. After washing, 50 μ l biotinylated monoclonal antibody HL857 was applied at 1 μ g/ml concentration to each well. After 1 hr incubation, the plates were washed and a 1:1000 dilution of strepavidin linked alkaline phosphatase (Sigma) was added to each well and incubated for 1 hr. After final washing, 100 μ l substrate was added to each well and incubated at room temperature. Optical density at 405 nm was read after 30 and 60 minutes of incubation in an automated ELISA plate reader. A duplicate plate on which no antigen was coated was also run to determine the amount of non-specific binding in the assay.

Once the optimal ELISA conditions were determined, known positive and negative control sera were titered out to a 1:1280 dilution. A blocking control plate, omitting the antigen coating step, was also run. This experiment was duplicated using sera that had been diluted in PBS with NaCl added to a final 0.5 M concentration. This added salt had been shown in previous experiments to reduce non-specific binding (Chapter 3). A similar experiment using Haplotrema antigen was also conducted to test for plasma cross-reactivity.

ELISA protocol for screening samples. A protocol was chosen for screening a large number of samples with limited antigen. The protocol used Maxisorp plates coated with 50 μ l

per well of adult Learedius antigen at 10 µg/ml concentration. Plasma samples were tested at a 1:10 dilution in 0.5 M NaCl-PBS, and optical density at 405 nm read at 30 minutes was used in analyses. All plasma samples were assayed at the same time to reduce assay variability. Plates included replicate positive and negative pool sera as well as blank controls.

Results

Plasma samples from 47 free-ranging lagoon turtles (48 samples including 1 recapture sample) and 57 reef turtles (58 samples including 1 recapture sample) were collected between 1992 and 1994. Body sizes of these turtles ranged from 26.8 to 73.4 straight carapace length and did not differ between sites (44.9 ± 9.5 reef and 42.3 ± 8.8 lagoon, Student's $t = 1.41$, $p > 0.10$). None of the reef turtles had GTFP. Twenty (40.4%) of the lagoon turtles had GTFP.

In addition, plasma from 46 captive-reared turtles was collected, including paired pre- and post-inoculation samples from 19 surviving turtles used in the 1993 transmission study. Plasma samples from the 1993 transmission study donors was also collected.

GTFP-Associated Herpesvirus

Histologic sections from GTFP biopsies containing herpesvirus intranuclear inclusions were uncommon, having been detected in only 7 (3.3%) of 210 biopsies by H&E and in only 18 (8.6%) by immunohistochemistry (see Chapter 6).

Therefore, the number of plasma samples that could be tested for antibody reactivity to herpesvirus was limited.

Free-ranging turtles. Plasma from 20 free-ranging GTFP-free turtles from the Wabasso Beach reef were tested and found to have negative (18) or very weak positive (2) antibody reactivity to herpesvirus inclusions. In contrast, all 20 plasma samples tested from GTFP-affected turtles from the Indian River lagoon showed strong antibody reactivity to herpesvirus inclusions. Thus GTFP-free turtles from a low GTFP prevalence site had only a 10% seroprevalence of detectable anti-herpesvirus antibodies while GTFP affected turtles from a high GTFP prevalence site had 100% seroprevalence of anti-herpesvirus antibodies. The association of herpesvirus immunoreactivity with clinical GTFP was statistically significant (Chi-Square = 32.7, $p < 0.001$).

Transmission study turtles. All 4 GTFP positive donors used in the 1993 transmission study were positive for antibodies to herpesvirus inclusions. Comparison of the pre-inoculation and 1 year post-inoculation plasma samples from captive-reared turtles used in the 1993 GTFP transmission experiment (Chapter 4) showed that seroconversion was associated with the induction of tumors (Table 7-1). None of the pre-inoculation plasma samples from these turtles had detectable immunoreactivity to herpesvirus inclusions. None of the 3 surviving sentinel turtles and none of the 4 treated

Table 7-1. Seroconversion to anti-herpesvirus immunoreactivity among green turtles with experimentally induced GTFP

Treatment ^a	GTFP Status ^b	N	No. with Anti-Herp Immunoreactivity ^c	
			Pre-bleed	Post-bleed ^d
Tumor Extract (Groups #1-3)	pos	12	0	12 (100%)
Tumor Extract (Group #4)	neg	4	0	0
Control Group	neg	3	0	0

^a Treatments and groups refer to the 1993 GTFP transmission study (Chapter 4).
^b Positive turtles developed tumors after inoculation with donor tumor homogenates.
^c Plasma diluted 1:10 showing specific intranuclear immunohistochemical reactions within foci of ballooning degeneration in target sections.
^d The post-bleed was collected approximately 1 year after inoculation.

but transmission negative turtles (from experiment #4) had seroconverted after 1 year. In contrast, all 12 turtles that developed GTFP following inoculation with tumor extracts from 3 separate donors had seroconverted, i.e., had detectable anti-herpesvirus immunoreactivity within 1 year post-inoculation. The association between immunoreactivity to herpesvirus and positive tumor transmission was statistically significant (Chi-Square = 18.7, $p < 0.001$).

Spirorchidiasis

Control turtles for ELISA development. Table 7-2 lists the findings from post mortem examination of 10 green turtles for whom ante-mortem plasma samples were available. The three captive (tank-reared) turtles were negative for gastrointestinal helminths, adult cardiovascular trematodes, and their ova. The seven free-ranging turtles had an abundant and diverse gastrointestinal trematode fauna with species representatives from several families including: Pronocephalidae, Paramphistomatidae, Plagiorchidae, Brachycoelidae, Angiodictyidae, Rhytidodidae, Gorgoderidae, Telorchiidae, and Calcooididae. Adult spirorchids were recovered from only 2 of these turtles. One turtle had only 2 adult Learedius learedii and the second turtle had only 6 adult Haplotrema sp. A third turtle (not listed) was the source of 45 adult Learedius used in antigen preparations.

Spleen digests revealed the presence of two types of spirorchid eggs in the free-ranging turtles. Type I ova

Table 7-2. Post-mortem diagnosis of trematode infections in green turtles used as controls in ELISA development

Spiroorchidiasis							
Identity	SCL (cm)	Location (Florida)	Adults	Type I ova	Type II ova	GI Flukes ^a	Status
TX91-#4	34.7	-	neg	neg	neg	neg	Captive- reared
TX91-#5	34.0	-	neg	neg	neg	neg	"
TX93-B3	35.3	-	neg	neg	neg	neg	"
BP2551	55.4	Indian River Co.	neg	neg	pos (rare)	pos	Free- ranging
BBA761	34.8	"	neg	neg	pos (rare)	pos	"
Bob	36.0	Monroe Co.	neg	neg	pos	pos	"
Billy	55.8	"	neg	pos	pos	pos	"
PPP157	43.5	"	neg	pos	pos	pos	"
Coastie	59.3	"	pos	pos	neg	pos	"
VMTH 105703	57.3	Broward Co.	pos	pos	neg	pos	"

^a Included species from one or more of the following trematode families: Angiodictyidae, Brachycoelidae, Calcooididae, Gorgoderidae, Paramphistomatidae, Plagiorchiidae, Rhytidodidae, and Telorchidae.

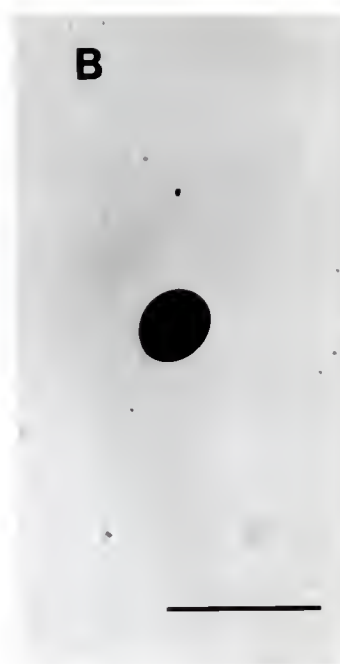


Figure 7-1. Spirorchid trematode ova recovered from Florida green turtles. (A) Type I ova measured 380-460 x 32-41 μm , were fusiform with bipolar extensions and conformed to descriptions of Learedius and Haplotrema ova. (B) Type II ova were small, 44-54 x 37-43 μm , and ellipsoid, and resembled Neospiroorchis ova. Unstained (scale bar = 100 μm).

measured 380-460 x 32-41 μm , were fusiform with bipolar extensions, and corresponded in morphology to Learedius or Haplotrema ova (Figure 7-1 A). Type II ova were ellipsoid and small, measuring 44-54 x 37-43 μm , and resembled those described for Neospiorchis species (Rand & Wiles, 1985; Wolke et al., 1982) (Figure 7-1 B). Two free-ranging turtles had only type I ova and 3 had only type II ova present in their spleens. The remaining 2 turtles had both types of ova in their spleen digests.

Plasma samples from the 3 captive-reared, helminth negative turtles were used as negative controls in the ELISA. Plasma samples from the two adult spirorchid infected turtles were used as positive controls. The other 5 turtles that were negative for adult spirorchids and Learedius-like type I ova, but positive for Neospiorchis-like type II ova and gastrointestinal trematodes were used to help assess the specificity of the ELISA.

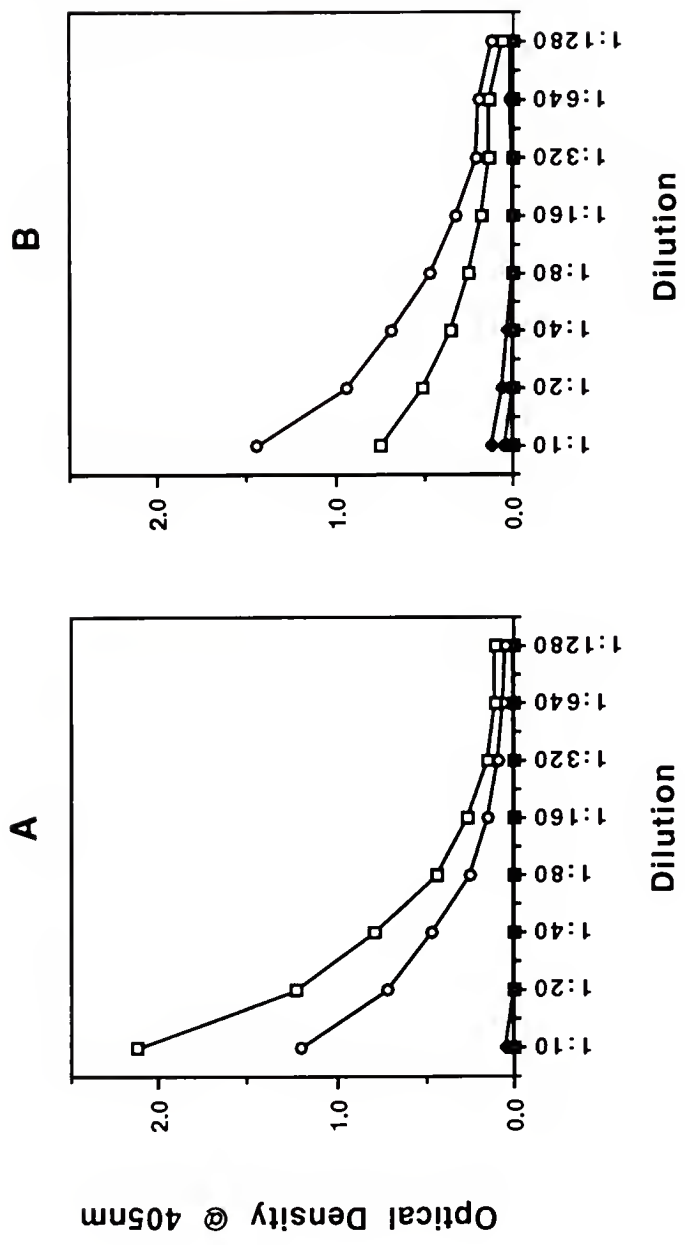
ELISA development. Antigen coating concentrations of 10 $\mu\text{g/ml}$ gave the largest difference between positive and negative control plasma optical density readings, so this coating concentration was used in the screening ELISA. Some plasma samples diluted in PBS had significant non-specific binding to ELISA plates coated with 0 mg/ml antigen, detectable even at high dilutions 1:160. Dilution of plasma in 0.5 M NaCl-PBS removed this non-specific binding. Figure 7-2 illustrates the ELISA results for serial dilutions of

control plasma. One positive control plasma had OD values exceeding the negative cutoff value ($\bar{x} + 3SD$) at 1:640 while the second was positive out to 1:320. Both turtles with active adult spirorchid infections had anti-spirorchid titers between 1:320 and 1:640, using either Learedius or Haplotrema crude antigen preparations (Figure 7-2A,B). Since, as far as is known, each of these turtles was infected with only a single species, it is apparent that there was considerable antigenic cross-reactivity between these spirorchid species.

The ELISA protocol for screening plasma samples only used crude antigen from Learedius because there was not enough Haplotrema antigen to test all samples. A plasma dilution of 1:10 for screening was chosen to minimize the chances of false negatives. The anti-Learedius ELISA results, optical density (OD) values read at 30 minutes, for plasma diluted 1:10 from the Learedius adult positive turtle and the Haplotrema adult positive turtle were 2.36 and 1.11 OD units respectively. When the ELISA was run using Haplotrema antigen, similar high OD values were found but the order was reversed. Thus it is apparent that either or both species can be used as antigen for ELISA.

ELISA results for the 3 confirmed negative turtles ranged from 0.028 to 0.087 ($\bar{x} = 0.06 \pm 0.03$). Using the mean + 3 SD as the cutoff value, samples were considered to be positive if $OD > 0.15$. When the results for 43 captive-reared presumed negative turtles, including the 19 transmission

Figure 7-2. Plasma 7S IgY antibody responses of control turtles to crude adult spirorchid antigen preparations. (A) Antibody responses to Learedius learedii antigen. (B) Antibody responses to Haplotrema sp antigen. Serial two-fold dilutions of plasma were tested against antigen coated at a concentration of 10 µg/ml. ELISA data presented are readings (OD_{405nm}) taken after 30 minutes incubation with substrate. Positive control samples were from turtles that were positive for adult Learedius, Coastie (open boxes), or adult Haplotrema, VMTH105703 (open circles). Negative control samples (solid symbols) were from 3 captive-reared trematode negative turtles (TX91-#4, TX91-#5, and TX93-B3).



study turtles, were examined they had a range of 0.006 to 0.150 OD units ($\bar{x} = 0.046 \pm 0.038$) with all values falling within the negative range established with the 3 proven negative turtles.

The 3 free-ranging turtles that were proven negative for spirorchid adults and type I (Learedius-like) ova but positive for type II (Neospiroorchis-like) ova and gastrointestinal trematodes had OD values ranging from 0.091-0.236 ($\bar{x} = 0.167 \pm 0.06$). Only the lowest OD value was within the negative range. Another plasma sample from this turtle, collected 7 months earlier, had an OD value above the negative cutoff value. These 3 turtles provided an intermediate OD range with an upper cutoff limit ($\bar{x} + 3SD$) of 0.35 for proven type I ova negatives. These low positive OD readings (> 0.15 but < 0.35) could be caused by cross-reactions with gastrointestinal trematode species or with spirorchid species, such as Neospiroorchis, that produce type II ova. Turtles that were positive for type II ova but negative for gastrointestinal trematodes, or spirorchid negative but gastrointestinal trematode positive, were not available, so it was impossible to distinguish between these 2 possibilities by ELISA. Among the 4 proven Learedius-like ova positive turtles, the lowest OD value was 0.476 which was above (1.4 times) the cutoff limit (0.35) for type I ova negative turtles.

ELISA survey of free-ranging green turtle plasma. Figure 7-3 shows the relative frequency distributions of the 2 population samples, Indian River lagoon and Wabasso Beach reef, across the 3 optical density range categories, discussed above, for 1:10 diluted plasma. Only 1 turtle from each location had an OD value < 0.15 . Using this value as the negative cutoff, seroprevalence estimates for spirorchid infection were 98% for the lagoon and 98.3% for the reef site. The sero-negative lagoon turtle had GTFP and obviously, there was no etiologic association between spirorchid exposure, as measured, and GTFP prevalence.

Using the higher negative cutoff value of 0.35, which was more specific for detecting anti-Learedius antibodies, the lagoon sample had a lower seroprevalence with 26 (56.4%) positives than the reef with 41 (72%) positives. However, the association of spirorchid seroprevalence with habitat was not statistically significant (Chi-Square = 3.1, $p > 0.05$).

Exclusion of turtles with GTFP from the habitat comparison (Figure 7-4) showed that more tumor-free lagoon turtles were spirorchid positive compared to reef turtles (78% and 72% respectively) but this difference was not statistically significant (Chi-Square = 0.32, $p > 0.50$). In contrast, there was a statistically significant difference (Chi-Square = 13.6, $p < 0.001$) between the anti-spirorchid antibody seroprevalence of tumor-bearing lagoon turtles (25%) and that of normal tumor-free reef turtles (72%).

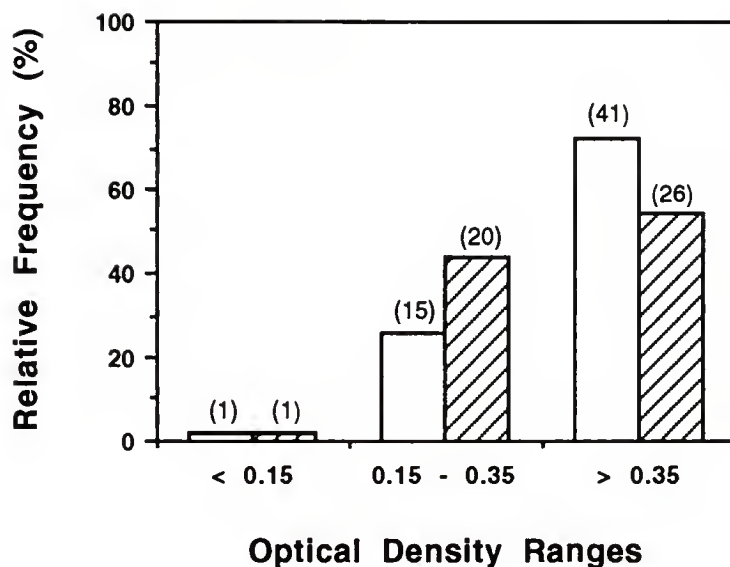


Figure 7-3. Relative frequencies of negative, intermediate, and positive 7S IgY antibody responses to Learedius learedii crude antigen in turtle plasma samples from two sites. Plasma samples diluted 1:10 were screened by ELISA in plates coated with 10 µg/ml of antigen. The range categories for 30 minute readings (OD_{405nm}) correspond to the following: trematode negative (< 0.15), adult spirorchid and type I ova negative but type II ova and gastrointestinal trematode positive (0.15-0.35), and adult spirorchid or type I ova positive (>0.35). Wabasso Beach reef samples (open boxes). Indian River lagoon samples (cross-hatched boxes) Numbers of turtles in each category are given in parentheses.

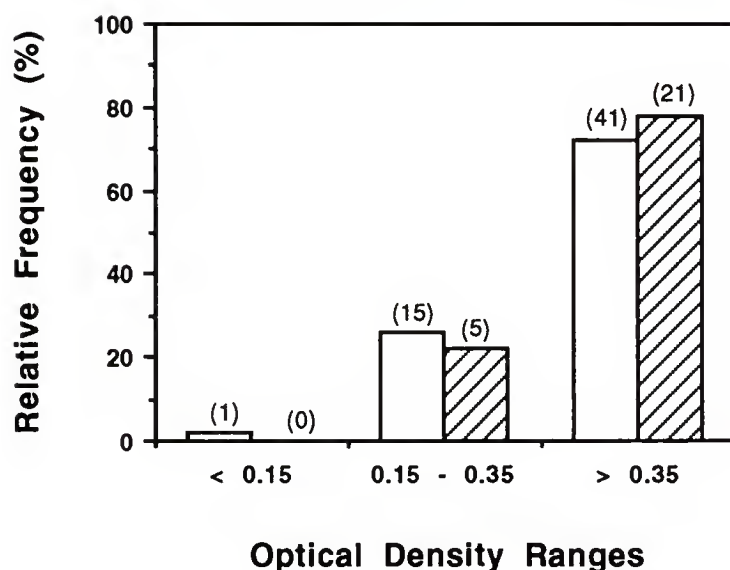


Figure 7-4. Relative frequencies of negative, intermediate, and positive 7S IgY antibody responses to Learedius learedii crude antigen in plasma samples from GTFP-free turtles from two sites. Plasma samples diluted 1:10 were screened by ELISA in plates coated with 10 µg/ml of antigen. The range categories for 30 minute readings (OD_{405nm}) correspond to the following: trematode negative (< 0.15), adult spirorchid and type I ova negative but type II ova and gastrointestinal trematode positive (0.15-0.35), and adult spirorchid or type I ova positive (> 0.35). Wabasso Beach reef turtles (open boxes). GTFP-free Indian River lagoon turtles (cross-hatched boxes). Numbers of turtles in each category are given in parentheses.

Only 5 (25%) of the 20 GTFP positive lagoon turtles were also spirorchid ELISA positive ($OD > 0.35$). In contrast, 21 (78%) of the 27 tumor-free lagoon turtles were spirorchid ELISA positive (Figure 7-5). Within the lagoon, of the 26 spirorchid ELISA positive turtles, only 5 (19%) were also GTFP positive. In contrast, 15 (71%) of 21 spirorchid negative lagoon turtles also had GTFP. The association between antibody reactivity against spirorchids, as measured, and tumor-free status within the lagoon sample was statistically significant (Chi-Square = 12.93, $p < 0.001$). These results do not support a positive association between spirorchidiasis and GTFP.

Discussion

GTFP-Associated Herpesvirus

The strong association between conversion to herpesvirus antibody reactivity and the development of experimentally induced tumors supports the hypothesis that the GTFP-associated herpesvirus is the etiologic agent of GTFP. Although these results cannot be taken to imply causation, at least these data suggest that conditions that are permissive to herpesvirus replication are linked to changes within GTFP epithelium. It is possible that the herpesvirus was co-transmitted along with the true GTFP-agent as a contaminant of the filtered tumor homogenates.

The strong association of detectable anti-herpesvirus antibodies with tumor status in free-ranging Florida green

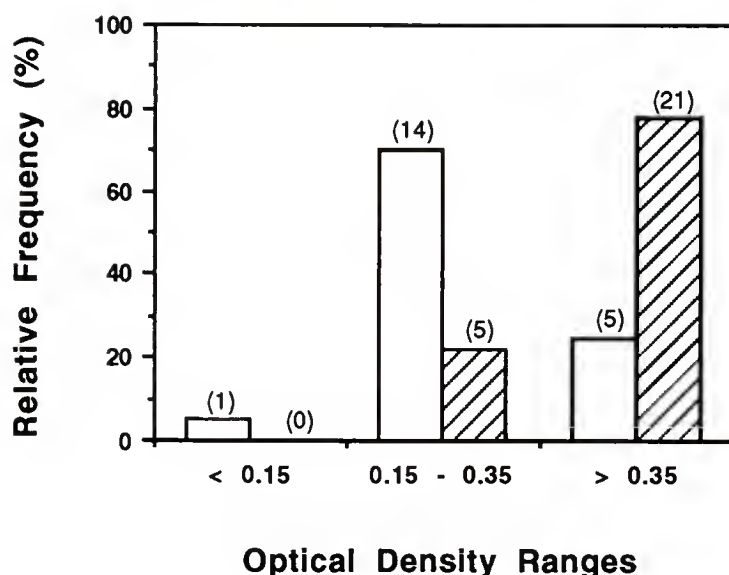


Figure 7-5. Relative frequencies of negative, intermediate, and positive 7S IgY antibody responses to Learedius learedii crude antigen in plasma samples from GTFP-positive turtles and GTFP-negative Indian River lagoon turtles. Plasma samples diluted 1:10 were screened by ELISA in plates coated with 10 µg/ml of antigen. The range categories for 30 minute readings (OD_{405nm}) correspond to the following: trematode negative (< 0.15), adult spirorchid and type I ova negative but type II ova and gastrointestinal trematode positive (0.15-0.35), and adult spirorchid or type I ova positive (> 0.35). GTFP-positive turtles (open boxes). GTFP-negative turtles (cross-hatched boxes) Numbers of turtles in each category are given in parentheses.

turtles, including the 4 transmission study donors, also suggests a causal relationship between herpesvirus and GTFP. Among free-ranging turtles, there was no possibility of nosocomial herpesvirus infection. Recently, detection of herpesvirus antibodies in free-ranging Hawaiian green turtles from a site with high GTFP prevalence adds further support to this hypothesis by demonstrating the widespread and coincident distribution of the virus with GTFP (Herbst & Balazs, unpublished data).

Determination of immunohistochemical reactivity to herpesvirus inclusions was qualitative and somewhat subjective. A more sensitive, specific, and objective diagnostic test is needed before extensive serologic surveys are conducted. Diagnostic test development for the GTFP-associated herpesvirus or other viral agents has been hampered by the limited availability of antigen. Successful isolation of this virus in culture or production of recombinant virus specific antigen would solve this problem. Until the virus is available for study, it will be impossible to determine whether the GTFP-associated herpesvirus is one of the two herpesviruses already described from green turtles (Rebell et al., 1975; Jacobson et al., 1986a), or a unique strain, family of strains, or a new species.

Spirorchidiasis

In contrast to the herpesvirus serology results, the association between immunoreactivity to spirorchid antigens

and clinical GTFP was either weak or strongly negative, depending on how the ELISA values were interpreted. Either way, the data do not support any hypotheses that involve spirorchidiasis in the etiology or pathogenesis of GTFP.

When all samples with OD values above the cutoff value (0.15) for trematode negative turtles were interpreted as positive for spirorchid exposure, i.e., assuming no false positives caused by cross-reactions with gastrointestinal species, nearly all (98%) juvenile turtles were positive and, consequently, there was no statistical association between spirorchid exposure and GTFP prevalence.

When the higher cutoff limit (0.35) was used, assuming OD values between 0.15 and 0.35 were false positives, then seroprevalence estimates ranged from 25% for GTFP positive lagoon turtles to 78% for tumor-free lagoon turtles. Although there was a statistically significant association between GTFP status and spirorchid antibody reactivity, the relationship was the opposite of that predicted by the hypothesis that tumors are caused by spirorchid ova (Harshbarger, 1984).

Results obtained by using the higher cutoff value (0.35) could be interpreted in several ways. Immunosuppression, causing a decline in spirorchid-specific humoral immune responses could explain the lower prevalence of high OD positive ELISA responses in GTFP-affected turtles. However, 1 of the adult positive turtles and 2 type I spirorchid ova

positive turtles had severe, debilitating fibropapillomatosis and yet had very high OD values. In addition, all GTFP-positive turtles had detectable antibodies to the GTFP-associated herpesvirus. Immunomodulation, following tumor induction, resulting in down-regulation of anti-spirorchid responses and up-regulation of anti-GTFP agent responses, could also explain these results. Another explanation is that the lagoon habitat does not support an active spirorchid life cycle, so that turtles moving into the lagoon would no longer get re-infected. Anti-spirorchid antibody titers would decline with time as resident turtles eliminated any infections they brought with them into the lagoon. Prolonged residency in the lagoon would also increase the probability of contracting GTFP if the lagoon is the reservoir for the infectious GTFP agent and associated cofactors (Ehrhart, 1991). This hypothesis could be tested by comparing plasma samples from long-term lagoon residents with those from turtles that recently entered the system, or by analyzing a series of samples from turtles held in pens within the lagoon. A serologic survey of green turtles from other lagoon habitats with lower GTFP prevalences may also help resolve this question.

If the lagoon habitat does not support completion of spirorchid life cycles then it becomes important to determine where and when green turtles become infected. It is possible that spirorchid infections are acquired by very young green

turtles in the pelagic environment during their 2-5 year pelagic existence. Small turtles 25-30 cm SCL have been documented with spirorchidiasis (Looss, 1902 cited in Lauckner, 1985) and the smallest turtle in this study (26.8 cm SCL) had antibodies to spirorchids. Sampling of pelagic juvenile green turtles will help resolve this question.

The ELISA described in this report was designed to detect 7S IgY antibodies to adult Learedius learedii crude antigen and provided a rapid non-destructive method to estimate the prevalence of spirorchid infection in turtle populations. However, there were limitations to data interpretation because of potential cross-reactivity with irrelevant antigens. On the one hand, cross-reactivity was useful because, although Learedius learedii is the most commonly reported species in Florida and Caribbean green turtles (Dyer et al., 1991; Greiner et al., 1980; Nigrelli, 1941; Rand & Wiles, 1985; Greiner, pers. comm.), it is not the only species found in fibropapillomas. The ELISA was able to detect antibody reactivity in plasma from a turtle infected with Haplotrema adults only, in plasma from 2 turtles whose tissues contained characteristic Learedius-like ova, and in plasma from 3 turtles whose tissues contained only Type II (Neosporchis-like) ova. Thus, the ELISA was sensitive enough to detect exposure to spirorchids where there were too few flukes to detect at necropsy or where the duration of exposure and time since clearance of infection

were unknown. In addition, the screening protocol was able to correctly classify plasma from 43 captive-reared and presumed negative turtles as negative (0% false positives).

On the other hand, potential cross-reactivity with gastrointestinal trematodes made it difficult to interpret OD values between 0.15 and 0.35. The 3 control turtles that had ELISA values within this range had been infected with type II ova producing spirorchids and gastrointestinal species. Because samples from turtles diagnosed with only one type of infection (type II spirorchid or gastrointestinal species) were not available, it remains unclear whether these values were diagnostic for spirorchidiasis. Similar problems with serologic cross reactivity between gastrointestinal trematodes and Schistosoma have been reported in mammals (Derouin et al., 1980; Hassan et al., 1989; Hillyer et al., 1980, 1988; McLaren et al., 1978).

The ELISA appeared to be sensitive and specific for antibodies to Learedius and Haplotrema (type I ova producing species) when the higher cutoff value (0.35) was used, since all 4 samples from turtles with proven exposure to type I species had OD values well above this limit. Additional plasma samples from turtles with well documented trematode exposures will be needed to evaluate this ELISA further. Studies are needed to identify antigens that are unique to spirorchid species for development of more refined serologic tests. Also, because antigenic cross-reactivity may vary

among spirorchid species, a battery of ELISA's, each using antigen from a different species, should be used for detection of green turtle antibody responses to spirorchidiasis.

Conclusion

The serologic data presented in this chapter are consistent with the hypothesis that the GTFP-associated herpesvirus, first reported by Jacobson et al. (1991) and subsequently observed in experimentally induced tumors, as well as additional spontaneous tumors (Chapters 4 & 6), is the cause of GTFP. While there is no doubt that spirorchid trematodes are important pathogens of green turtles and that concurrent spirorchidiasis may influence the clinical course of GTFP, the serologic data presented here support earlier conclusions (Chapter 6) that the presence of spirorchid ova in tumors is an incidental finding. A clearer understanding of the epizootiology of herpesvirus and spirorchid infections in green turtle populations must await further studies with improved serodiagnostic assays.

CHAPTER 8
ESTABLISHMENT OF CELL LINES AND PRELIMINARY STUDIES OF
DIFFERENTIAL GENE EXPRESSION BETWEEN NORMAL AND TUMOR-DERIVED
FIBROBLASTS

Introduction

Cutaneous fibropapillomas and associated visceral fibromas of green turtles are histologically benign tumors composed of well-differentiated cells with few mitotic figures and no anaplastic changes (Chapter 2). These histologic findings are corroborated by electron microscopic studies of cultured fibroblasts derived from tumors (Mansell et al., 1989) and flow cytometry (Papadi et al., in press). The apparently normal cytologic features of tumor fibroblasts raises the question of whether or not these cells are transformed (neoplastic).

It is obvious from histopathologic examination of spontaneous and experimentally induced GTFP (Chapter 6) that the infectious GTFP agent causes fibroblast proliferation. However, the mechanism is not understood and may involve either direct infection and transformation of fibroblasts by the agent or paracrine stimulation of uninfected fibroblasts by adjacent infected epithelium. Although the GTFP-associated herpesvirus may explain the epidermal changes observed in

cutaneous GTFP, the pathogenesis of dermal fibroblast proliferation has not been elucidated.

Studies to understand the nature of proliferating GTFP fibroblasts require methods to distinguish benign neoplastic cells from hyperplastic normal cells. Phenotypic alterations in vitro, such as loss of contact inhibition (transformation foci), decreased dependence on exogenous growth factors found in serum, anchorage independence (cloning in suspension), and differences in growth rate or confluent density, can be used to detect the transformed (neoplastic) phenotype. Cellular transformation can be detected in vivo by tumor formation in an animal model. With appropriate methods to distinguish phenotypes, comparative studies are possible.

This study was an attempt to show that GTFP fibroblasts are neoplastic, i.e., genetically different from normal fibroblasts, by demonstrating phenotypic differences between tumor-derived and normal skin-derived cell lines. In addition, preliminary studies of gene expression were initiated.

Materials and Methods

Cell Lines

Three wild green turtles with spontaneous GTFP, 11 turtles with experimentally induced GTFP (Chapter 4), and one clinically normal turtle were used as sources of cell lines. Turtles were anesthetized and prepared for aseptic surgery. Fibropapillomas were resected, washed extensively in sterile

saline, and minced in D-MEM/F12 with 240 U/ml Penicillin G, 240 µg/ml Streptomycin sulphate, and 0.6 µg/ml Amphotericin B (2.4 x Antibiotic-Antimycotic, GIBCO, Grand Island, NY, USA) and transported on ice to the laboratory. Multiple 6 mm punch biopsies of normal turtle skin were collected and prepared in the same way.

Tissue samples were placed in sterile 50 ml centrifuge tubes and washed 3 times in Hank's Balanced Salt Solution (HBSS, GIBCO). The HBSS was then replaced by HBSS containing 300-600 U/ml collagenase (CLS-2, Worthington Biochemical Corp., Freehold, NJ, USA) and incubated at 30°C. Disaggregated cells were harvested periodically by pipetting the digest solution into 10 volumes of ice cold D-MEM/F12 with 10% fetal bovine serum (FBS). Cells were pelleted at 400 x g for 10 minutes at 4°C and resuspended in D-MEM/F12 plus 10% FBS. Digestions were carried to completion (usually 12-24 hrs) and all cells were pooled, counted, and seeded into plastic culture flasks at a density of 1×10^5 cells per ml and incubated at 30°C in a 5% CO₂ atmosphere. Cells were grown to confluence and then collected by treating the flask with 0.25% trypsin-1 mM EDTA in HBSS. Aliquots of primary digest cells were cryopreserved in D-MEM/F12 containing 5% DMSO. Primary cell cultures or early passage (up to TC4) were used in experiments.

In Vitro Assays

Two cell lines, 1 GTFP derived (Coastie FP) and 1 normal skin derived (Everglades NSF) were studied.

Serum dependence. The ability of cultures to grow in reduced serum concentrations was tested. Cells from each cell line were seeded, 5×10^5 cells per well, into 6-well plates and cultured in unsupplemented D-MEM/F12 or in media supplemented with various concentrations of FBS ranging from 1.25 to 5%. Cultures were incubated at 30°C and cells were harvested and counted after 3 and 6 days in culture.

Agar cloning. Preliminary attempts were made to clone tumor cells in agar. In 6-well plates between 1×10^3 and 4×10^4 cells per well of each cell line were suspended in a final concentration of 0.33% agar (a 2:1 mixture of SeaPlaque® and SeaKem® agars, FMC BioProducts, Rockland, ME, USA) in D-MEM/F12 and laid over a 1% agar base. Cultures were incubated at 30°C and monitored for colony formation for up to 1 month.

Transformation focus formation. Confluent cell cultures were maintained in growth medium (D-MEM/F12 plus 10% FBS) and re-fed 2-3 times a week for several weeks and observed for cell overlap and piling up.

Tumorigenicity Assay (In Vivo)

Experiments were conducted to determine whether GTFP-derived fibroblasts could form tumors in one of the established laboratory animal models, congenitally athymic (nude, nu) or severe combined immunodeficiency (scid) mice.

Mice. C57BL/6J-nu/nu, C.B17-scid/scid, and NOD-scid/scid were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in pairs on hardwood chips in polycarbonate microisolator cages (12 1/2 x 9 1/4 x 6 inches) fitted with filter tops. Mice were fed autoclaved rodent chow (Ralston Purina, St. Louis, MO, USA) and provided with sterile water containing 1 mg/ml sulfaquinoxaline (Ralston Purina, Sulfa-Nox Liquid). The mouse room was maintained on a 12:12 light cycle, temperature $21 \pm 1^{\circ}\text{C}$.

Inoculations. In preliminary experiments, GTFP-derived cells were injected subcutaneously into the flank, footpad, and medial margin of the pinna (ear). Later experiments used ear inoculations only. Aliquots of 2×10^6 to 5×10^6 cells suspended in 100 μl PBS were injected. Mice were observed weekly for at least 4 months for evidence of tumor development. Mouse tumors were examined by immunohistochemical methods and karyotyped to identify their species of origin.

Immunohistochemistry. Mouse-passaged fibromas were tested with a polyclonal mouse immune serum against green turtle tissue. Briefly, 5 μm sections of 10% formalin-fixed, paraffin embedded tissue were deparaffinized and rehydrated through a graded alcohol series. Sections were treated with 3% H_2O_2 to block endogenous peroxidase activity, and then treated with 0.125% trypsin-0.1% CaCl_2 in PBS for 20 minutes

at 37°C to recover antigenicity. After washing for 30 minutes with 3 changes of PBS the sections were incubated for 2 hours with dilutions (1:100, 1:500, or 1:1000) of either normal BALB/c serum or immune serum produced in BALB/c mice by repeated inoculation with GTFP homogenate in RIBI's adjuvant. Mouse antibody binding was detected using a horseradish peroxidase conjugated avidin-biotin complex kit, following manufacturer's instructions (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). Slides were incubated in substrate (3,3' diaminobenzidine, 0.3 mg/ml in PBS) and the color reaction monitored in control slides.

Karyotyping. Mouse passaged fibromas were collected under aseptic conditions, washed in sterile HBSS and disaggregated in collagenase as described above. Cells were seeded into culture flasks, grown to approximately 70% confluence, and submitted to the Cytogenetic Laboratory, University of Florida for karyotype determination. Harvest of metaphase cells was performed by arresting with exposure to demecolcine (Colcemid®, Sigma Chemical Co., St. Louis, MO, USA) (1 µg/ml final concentration) for 45 minutes, followed by brief enzymatic (trypsin-EDTA) release of attached cells. Cells were treated with 0.075 M KCl hypotonic solution for 20 minutes at room temperature and fixed in 3:1 methanol/acetic acid. Slides were prepared following standard chromosome spreading techniques. Chromosomes were treated with trypsin and stained with Geimsa, following standard human chromosome

banding techniques, to produce a G-banding (GTG) pattern (Seabright, 1971). Karyotypes were constructed via computer-imaging and homologous pairs were arranged in descending size order.

Detection of GTFP Cell-Associated Antigens

Once methods were established to distinguish tumor-derived fibroblasts from normal cells, preliminary studies were conducted to identify tumor cell-associated antigens for use in diagnostic applications and to help identify the etiologic agent.

Indirect immunofluorescence was used to detect antigens that may be expressed in GTFP cells. Plasma samples from 4 GTFP-positive turtles that had been chronically immunized with formalinized autogenous tumor homogenates were used for this purpose. Paired cell lines (A-1 normal and A-1 GTFP) were grown to confluence in chamber slides (Lab-Tek®, NUNC, Naperville, IL) in D-MEM/F12 plus 10% FBS. Slides were washed with serum-free media, air-dried, and fixed for 30 minutes with 70% ethanol. The slides were then incubated for 30 minutes at 4 °C with a 1:10 dilution of plasma from one of 4 GTFP-positive immunized turtles or from a clinically normal unexposed turtle. After washing for 15 minutes in three changes of PBS, slides were incubated with a mixture of monoclonal antibodies HL 673 and HL857 (1 µg/ml each) for 30 minutes at 4°C. This was followed by washing and incubation with FITC-conjugated sheep anti-mouse Fab' (1:200 dilution).

Slides were washed in PBS, mounted, and examined under fluorescence microscope to detect any differential staining between normal and tumor derived cells.

A similar experiment was conducted to detect cell surface antigens (membrane fluorescence), using trypsin-EDTA treated suspensions of 1×10^6 normal and tumor-derived fibroblasts as targets.

Detection of GTFP-Associated Changes in Gene Expression

Preliminary studies to detect differences in gene expression between normal and tumor-derived fibroblasts were begun using differential message display (Liang & Pardee, 1992)

Cell lines and RNA extraction. Two pairs of matched tumor and normal skin derived fibroblast cell lines (A-1 and D-1) were expanded to approximately 6×10^7 cells. Cells were allowed to reach confluence prior to extraction. Total RNA was extracted from cells using an extraction kit (Stratagene RNA Isolation Kit Cat # 200345, Stratagene, LaJolla , CA, USA) based on the method of Chomczynski and Sacchi (1987). Briefly, for each cell line cells were detached from their flasks with trypsin-EDTA, washed in HBSS, and counted. An aliquot of 5×10^6 cells of each cell line was inoculated into scid/scid mouse ears to confirm the phenotype at the time of RNA extraction. Aliquots containing 3×10^7 cells were pelleted at $500 \times g$ for 10 minutes at 4°C . The pellets were immediately resuspended in ice cold denaturing solution (5.7

M guanidinium isothiocyanate with 7.2 mM final concentration β -mercaptoethanol added) at a working volume of approximately 500 μ l per 1×10^6 cells. To this suspension was added one-tenth volume of 2 M sodium acetate (pH 4.0) followed by water-saturated phenol in a volume equal to the denaturing solution volume and chloroform:isoamyl alcohol mixture equal to one fifth the volume of denaturing solution. The suspension was then vortexed vigorously for 5 minutes and then allowed to stand on ice for 15 minutes. The mixture was then centrifuged for 20 minutes at 10,000 rpm at 4°C. The upper aqueous phase was removed with care taken to avoid the interface. Total RNA was precipitated from the aqueous phase with a volume of isopropanol at -70°C overnight. The precipitate was then pelleted and resuspended in 1/3 original volume of denaturing solution and precipitated with 1 volume of isopropanol. The precipitate was then pelleted, washed in ethanol, and dried. The pellet was resuspended in DEPC treated water to which 1/10th volume of 3 M sodium acetate (pH 5.2) was added followed by 2 x volume of 100% ethanol. The precipitate was pelleted, washed, air dried, and resuspended in 200 μ l water. Recovery was monitored by the ratio of absorbances ($OD_{260}:OD_{280}$).

Differential message display. Total RNA extracts from 2 matched cell lines were submitted to Dr. Ratna Chakrabarty (University of Florida, Gainesville, FL) for the initial stages of differential message display (Liang & Pardee,

1993). Briefly, the RNA preparations were treated with RNase-free DNase to remove possible chromosomal DNA contamination. The cDNAs of a subset of total mRNA were produced by reverse transcription using 4 sets of degenerate anchored (3') primers (T12MN) where M is G, A, or C and N is G, A, T, or C. With 12 possible combinations of the last 2 bases, each primer recognizes 1/12th of the total mRNA population.

Partial cDNA sequences were amplified using 5' end primers, corresponding 3' end primers, and labelled dATP such that 50-100 cDNAs were amplified. The 5' primers were arbitrary decamers allowing annealing positions to be randomly distributed in distance from the polyA tail. The 5' primers were designed to maximally randomize the 3' end with a fixed 5' end.

Following amplification, short 100-500 bp cDNA sequences were labelled and separated on a sequencing gel. The tumorigenic and normal cell products were run on adjacent lanes allowing side-by-side comparison of the mRNA expression pattern of tumorigenic versus normal skin fibroblasts. Bands that are present in one cell line but absent in the other were marked for further study.

Results

Cell Lines and In Vitro Characteristics

Twenty four green turtle fibroblast lines were successfully established and included 12 GTFP cell lines derived from 9 cases of experimentally induced and 3 cases of

spontaneous GTFP, 11 cell lines derived from normal skin of GTFP-affected turtles, and 1 cell line derived from an unaffected turtle (Table 8-1). There were 9 pairs of matched fibroblast lines (GTFP-derived and normal skin-derived from the same individual). Primary cell lines from some individuals failed to become established due to fungal contamination.

Tumor-derived fibroblasts were morphologically indistinguishable from normal fibroblasts under light microscopy and grew to similar confluent densities (Figure 8-1A and B). Preliminary serum limitation experiments failed to show any difference between normal and GTFP derived lines (data not shown). Similarly, transformation foci were not observed and preliminary attempts to clone GTFP-derived cells in soft agar were unsuccessful.

Tumorigenicity of GTFP-Derived Cell Lines

Preliminary experiments, in which 2 tumor-derived cell lines were inoculated into flank, footpad, or medial ear margin of 2 C.B17-scid/scid mice, resulted in fibroma growth in the ear and footpad, but not in the flank, after at least 6 months observation. In subsequent experiments, 12 GTFP-derived fibroblast lines and 12 normal fibroblast lines were tested for tumorigenicity in the ears of 1 or more scid/scid or nu/nu mice. While none of the 12 normal skin-derived fibroblast lines caused tumors after more than 4 months observation, all tumor-derived fibroblast lines caused tumors

Table 8-1. Tumorigenicity of fibroblast lines derived from green turtles

Cell line	Source	Tumor Production		
		C.B17- scid/scid	NOD- scid/scid	C57BL/6J- nu/nu
TX91-#5 FP	Induced GTFP	1/1	nt	nt
TX93-A1 FP	"	2/2	1/1	nt
TX93-A2 FP	"	nt	1/1	nt
TX93-A3 FP	"	nt	1/1	nt
TX93-B3 FP	"	nt	1/1	nt
TX93-C2 FP	"	nt	1/1	nt
TX93-C3 FP	"	nt	1/1	nt
TX93-D1 FP	"	1/1	1/1	nt
TX93-D2 FP	"	nt	1/1	nt
Coastie FP	Spontaneous GTFP	2/2	nt	1/3
Everglades FP	"	2/2	nt	0/1
Flamingo FP	"	2/3	nt	1/1
Jackie NSF	Normal Skin	0/3	0/1	0/3
TX93-A1 NSF	"	0/1	nt	nt
TX93-A2 NSF	"	nt	0/1	nt
TX93-A3 NSF	"	nt	0/1	nt
TX93-B2 NSF	"	nt	0/1	nt

Table 8-1--continued.

Cell line	Source	Tumor Production		
		C.B17- scid/scid	NOD- scid/scid	C57BL/6J- nu/nu
TX93-B3 NSF	"	nt	0/1	nt
TX93-C1 NSF	"	0/1	nt	nt
TX93-C2 NSF	"	nt	0/1	nt
TX93-C3 NSF	"	nt	0/1	nt
TX93-D1 NSF	"	nt	0/1	nt
TX93-D2 NSF	"	nt	0/1	nt
Everglades NSF	"	0/1	0/1	nt

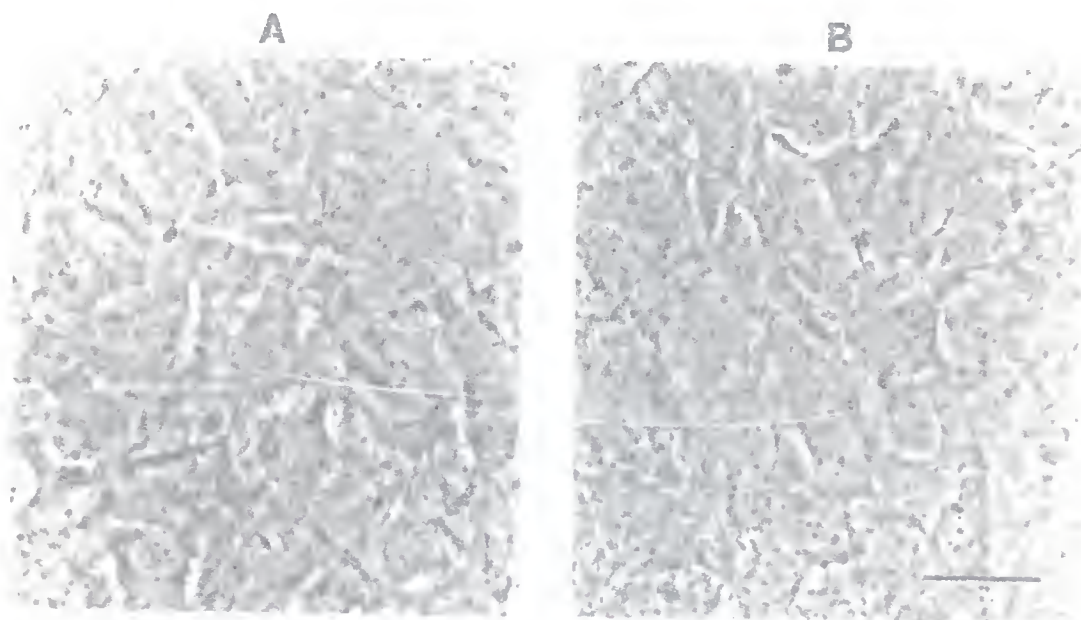


Figure 8-1. Green turtle fibroblast cultures. Confluent monolayers of early passage GTFP-derived fibroblasts (A) and normal skin-derived fibroblasts (B) from green turtle TX93-D1 showing similar morphological appearance in vitro. Phase contrast (scale bar = 50 μ m).

in one or more immunodeficient mice (Table 8-1). Tumors (Figure 8-2) were produced more reliably in scid/scid mice (12 of 12 lines tested, 18 of 19 mice injected) than in nu/nu mice (2 of 3 lines tested, 2 of 5 mice injected).

Visible tumors 1-2 mm in diameter were first noted between 5 and 44 weeks post inoculation ($\bar{x} = 12.4 \pm 12.3$ weeks). This range probably results from variation in actual dose of cells received and from difficulties in detecting early tumors which could be obscured by hair or skin folds.

Fibromas derived from mouse ears had positive immunohistochemical reactions with mouse anti-GTFP immune serum but not with normal mouse serum. Positive staining was found in stromal cells (fibroblasts) but not in endothelium or blood cells within tumors. Normal mouse dermis and subcutis adjacent to fibromas did not show immunoreactivity (Figure 8-3).

Karyotypes (Figure 8-4) of cell lines re-derived from mouse ear fibromas were typical for Chelonia mydas (modal 2N = 55) (Koment & Haines, 1982; Makino, 1952), as opposed to mouse (2N = 40), confirming the origin of these tumors.

GTFP Cell-Associated Antigens

Indirect immunofluorescence staining of normal and tumor derived fibroblasts using immune serum from GTFP-positive turtles failed to reveal any differential staining of cells. Weak background staining was detected with both immune and normal control turtle plasma, possibly due to inadequate

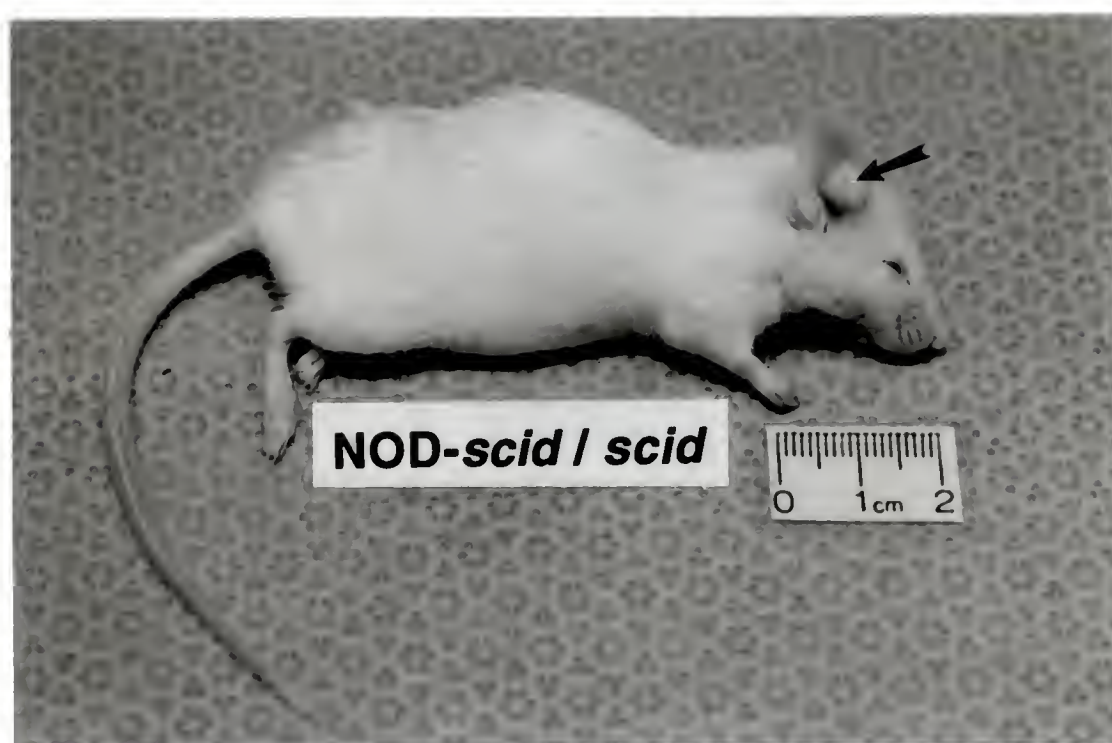


Figure 8-2. Tumorigenicity of GTFP-derived fibroblasts in immunodeficient mice. NOD-scid/scid mouse with fibroma (arrow) that developed within 2 months following injection of 5×10^6 early passage GTFP-derived fibroblasts into the medial margin of the pinnae (ear).

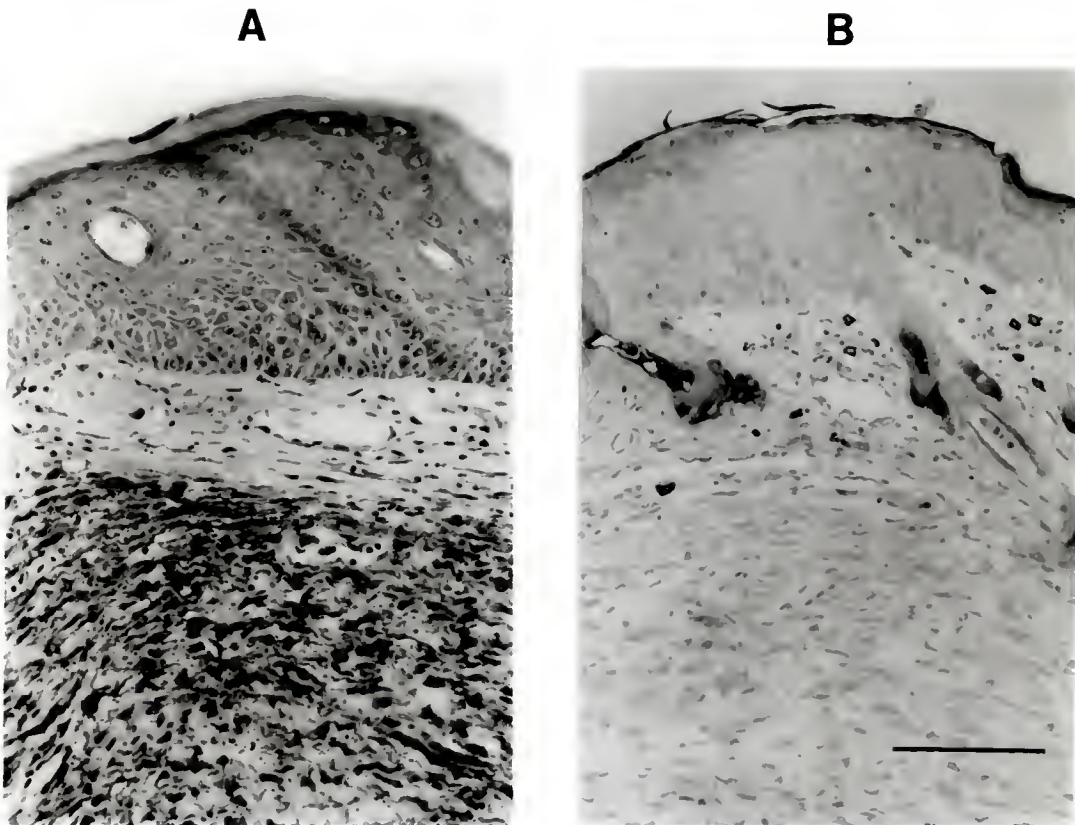


Figure 8-3. Immunohistochemical detection of green turtle fibroblasts in mouse ear fibromas. Histologic sections, from fibromas that were induced in mouse ears by injection of early passage GTFP-derived fibroblasts, were incubated with dilutions of serum from BALB/c mice that were immunized with GTFP homogenates or from unimmunized mice. Binding of mouse antibodies was detected with a horseradish peroxidase-conjugated avidin-biotin complex kit. (A) Antiserum diluted 1:100 from immunized mice showed specific immunoreactivity with tumor fibroblasts but not with surrounding mouse dermis. (B) Normal mouse serum diluted 1:100 showed no specific immunoreactivity. (Scale bar = 100 μ m).

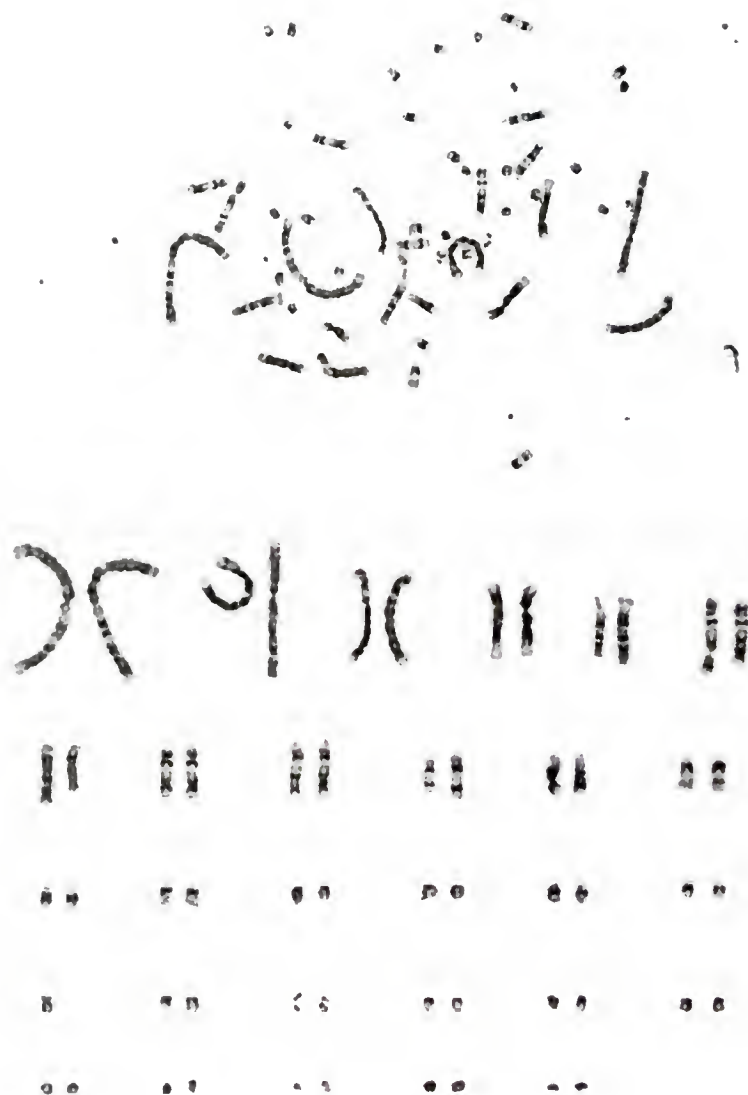


Figure 8-4. Chromosomes of fibroblasts derived from mouse ear fibromas. Typical Geimsa banded chromosome spread (top) and karyotype (bottom) of primary fibroblast cultures derived from fibromas induced in immunodeficient mice by injection of $2-5 \times 10^6$ early passage GTFP-derived fibroblasts into the medial margin of the ear. The karyotype (modal $2N = 55$) confirmed that these cells were of green turtle origin.

washing or non-specific binding of reagents to fibroblasts. On the other hand, the mouse anti-GTFP immune serum, used as a positive control showed strong fluorescence on both normal and tumor-derived cell types.

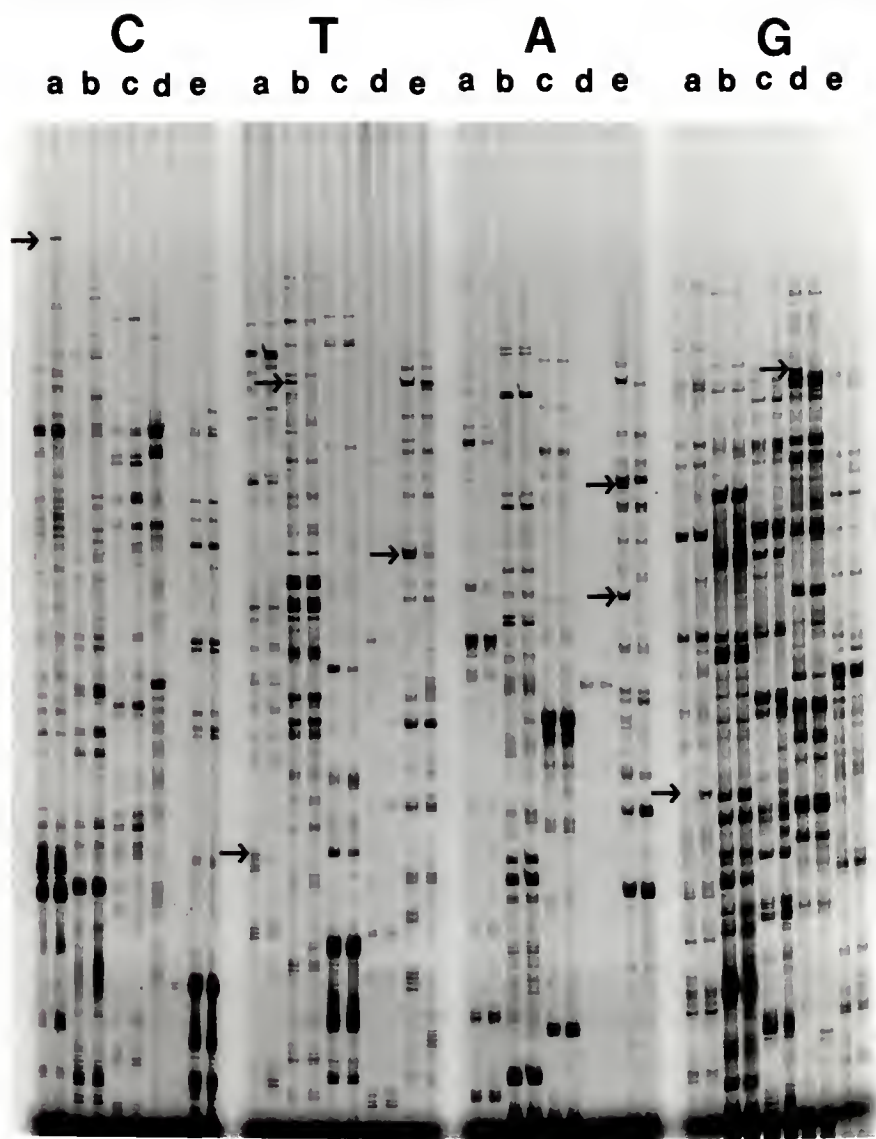
GTFP-Associated Changes in Gene Expression

Preliminary studies of matched normal skin and GTFP fibroblast lines, derived from the same individual and propagated under identical culture conditions, were performed to identify differences in messenger RNA expression between them. The tumor-derived cell line was confirmed to be tumorigenic by successful fibroma formation in mouse ears that were inoculated at the time of RNA extraction. Normal skin-derived fibroblasts inoculated at the time remained non-tumorigenic. Side-by-side comparison of the size patterns of labelled amplified cDNA sequences for 20 primer combinations (shown in Figure 8-5) revealed several clear differences, including at least 6 bands that were present in tumor but absent in normal cells, and at least 2 bands that were present only in normal cells.

Discussion

Investigation of the molecular pathogenesis of fibroblast proliferation characteristic of GTFP depends upon the development of methods to distinguish putative transformed cells from normal cells. In this study GTFP-derived fibroblasts were not readily distinguishable from normal skin-derived fibroblasts by morphology and growth

Figure 8-5. Differences in gene expression between normal skin-derived and tumorigenic GTFP-derived fibroblasts. Differential message display (after Liang & Pardee, 1992) was used to compare the pattern of mRNA expression between two matched fibroblast lines (normal skin-derived and tumorigenic GTFP-derived) cultured from a turtle (TX93-A1) with experimentally-induced GTFP. Twenty primer combinations (4 anchored 3' primers and five 5' primers) were used to amplify, by PCR, a number of short cDNA sequences from each cell line. These short amplified sequences were labelled and separated by size on a sequencing gel. Each pair of lanes is a side-by-side comparison of amplified cDNA sequences from GTFP fibroblasts (on the left) with those from normal skin fibroblasts (on the right) for a given primer combination. The 3' primers, labelled C, T, A, or G correspond to the sequences T12CN where N is either C,T,A, or G. The 5' primers (labelled a,b,c,d, or e) were arbitrary decamers designed to have a fixed 5' end and maximally randomized the 3' end. Examples of bands that are differentially expressed are indicated by arrows.



characteristics in vitro. These results corroborate a previous study (Mansell et al., 1989) that described 2 fibroblast lines derived from green turtle fibropapillomas as having normal in vitro morphology, although fibroblast lines from normal skin were not examined. This study clearly demonstrates, however, that GTFP-derived fibroblasts are qualitatively different from normal because they produce tumors in vivo. These findings support the hypothesis that GTFP fibroblasts are transformed (neoplastic) cells rather than normal cells responding to hyperplastic stimuli. The fact that other phenotypic indicators of transformation were not exhibited by GTFP fibroblasts suggests that these cells have relatively few cellular alterations. In this respect, GTFP fibroblasts resemble keloid-derived fibroblasts in humans, which are also tumorigenic in immunodeficiency mice but otherwise exhibit relatively subtle differences from normal fibroblasts (Estrem et al., 1987).

Congenitally athymic (nude, nu) and severe-combine immunodeficiency (scid) mice have proven to be valuable models for tumorigenicity studies of human cancers (Phillips et al., 1989; Williams et al., 1993). However, the mouse's high body temperature has made it a poor model for transplantation of poikilothermic vertebrate tissues. For example, although Manning et al. (1973) were able to demonstrate engraftment of skin from several reptiles and amphibians into nude mice, the grafts were not

morphologically normal, a possible result of the suboptimal thermal environment. The failure of GTFP cells to engraft in the flanks of mice in this study also supports this conclusion.

Few experimental systems have been developed for assaying tumorigenicity of lower vertebrate cells. Lucké renal adenocarcinoma cells have been cultured in the anterior chamber of the eye of Rana pipiens (Mizell, 1969). Rausch and Simpson (1988) developed a model system using irradiated Anolis carolinensis for in vivo tumorigenicity studies. Irradiated lizards were able to maintain grafts for up to 2 months. Limitations on husbandry conditions and the lack of "clean" barrier reared animals makes this model difficult to use. In addition, for very slow growing tumors such as GTFP, the lizards may recover their immune competence before the tumors become clinically apparent.

This study has demonstrated that immunodeficiency mice can be used in tumorigenicity assays of cells derived from poikilothermic (lower) vertebrates if inoculations are performed at cooler anatomic sites such as the footpad or pinna. The mouse ear tumorigenicity model is practical because the mice can be readily incorporated into standard laboratory animal facilities with microisolator caging systems. A disadvantage of the mouse model is that it is unlikely to be useful for studies of metastasis.

The availability of proven tumorigenic cell lines derived from fibropapillomas opened the possibility of using cultured GTFP fibroblasts as target antigens in the development of immunodiagnostic tests and assays of green turtle anti-tumor immune responses. Preliminary tests were designed to determine if green turtles that had been chronically exposed to GTFP, through natural infection and vaccination, had developed antibodies against GTFP fibroblasts. Initial results indicated that green turtles do not mount a humoral immune response to tumor cells. These findings suggested that either, transformed fibroblasts were not expressing any unique tumor-associated antigens (e.g. viral antigens on their surfaces), or that these unique antigens did not elicit a detectable antibody response in the green turtles used in this study. Assuming that fibroblasts are not permissive for virus replication (see Chapter 6) and do not express viral antigens on their surface it is not surprising that they would not elicit an antibody response. These results are in contrast to reports from other virus-induced fibroma cell lines where unique tumor-associated antigens have been detected with convalescent serum (Barthold & Olson, 1974, 1978). Although, green turtle antibodies to tumor cells were not detected it is still possible that unique agent-specific or tumor-associated antigens and peptides are expressed on the cell surface, possibly in association with MHC class I molecules. Further studies,

including studies of cell-mediated immune responses to GTFP-derived fibroblasts are needed.

The availability of matched tumorigenic and normal fibroblast lines has made it possible to study the molecular pathogenesis of cellular transformation in GTFP. Preliminary work has shown several obvious changes in gene expression that are associated with the tumorigenic phenotype. These differentially expressed mRNA sequences now must be verified by Northern blot analysis, cloned, sequenced, and if possible identified. Although some of these differentially expressed bands may prove to be artifacts, and others may represent up-regulation of host cell gene expression, one or more of these products may represent the transcription of GTFP agent-specific genes. Once the full GTFP agent-specific gene sequences are identified, these could be used to probe nucleic acid sequences isolated from tumors, transmission positive tumor extracts, or purified virus particles to help identify the causative agent. Transformation experiments could also be performed with the gene sequences that are identified.

CHAPTER 9

SUMMARY AND RECOMMENDATIONS

The studies described in this dissertation have helped to elucidate the nature of GTFP and lay the groundwork for identifying the causative agent, for producing practical diagnostic tests with which to distinguish exposed from unexposed turtles, and for understanding the pathogenesis of this disease at the molecular level.

The production of a series of monoclonal antibodies, specific for green turtle antibody classes, described in chapter 3, provided the reagents needed to detect turtle humoral immune responses to potential pathogens that may cause GTFP and tumor-associated antigens. Development and refinement of practical serodiagnostic tests for detecting green turtle antibody responses to GTFP-associated antigens will be possible, once the relevant antigens become available.

The transmission studies (Chapter 4) conducted over several years have demonstrated conclusively that GTFP is an infectious disease caused by a filterable subcellular agent. This agent is necessary and sufficient to cause tumors, although other factors may play a role in modulating the

onset and severity of GTFP, or the capability of affected animals to recover from infection.

The identity of the GTFP agent remains unclear. There is now, however, a substantial amount of circumstantial evidence to support a herpesvirus etiology. A herpesvirus was the only virus identified by electron microscopy within spontaneous and experimentally-induced fibropapillomas (Chapter 4). Preliminary characterization experiments (Chapter 5) showed that the GTFP agent is chloroform sensitive, which is consistent with it being an enveloped virus, such as a herpesvirus. Except for fibroblast transformation, the histopathologic features described for GTFP, can be explained by classic herpesvirus pathogenesis, and herpesvirus antigens can be detected in tumors by immunohistochemistry (Chapter 6). Lastly, serologic evidence of exposure to herpesvirus is common among GTFP-affected turtles (Chapter 7).

Nonetheless, as discussed previously, a number of viruses may induce tumors in tissues that are not permissive for virus replication, so it remains possible that GTFP is caused by an as yet unidentified virus that is not shed from tumors. Examples of this type of viral oncogenesis include Cottontail Rabbit Papillomavirus induced papillomas in domestic rabbits (Ito & Evans, 1961), Bovine Papillomavirus induced sarcoids in horses (Amtmann et al., 1980; Angelos et al., 1991; Lancaster et al, 1977), Epstein-Barr virus and Marek's disease virus induced lymphomas in humans and poultry

(Henle & Henle, 1985; Powell, 1985), and Avian Sarcoma Virus induction of sarcomas in poultry (Benjamin & Vogt, 1990). The situation is complicated by the propensity for latent herpesvirus infections to recrudesce in tumor tissues and in stressed animals.

On the other hand, transmission experiments showed that the agent is present in an infectious form in spontaneous tumors. Experimental transmission by direct inoculation (s.c., i.d., or scarification), however, may favor idiosyncratic forms of transmission such as transfection with transforming DNA sequences. Several viruses, including herpesviruses and papillomaviruses, are known to have DNA that is infectious under experimental conditions (see Chapter 4). These specialized routes of transmission are unlikely to explain how transmission occurs in nature. That is, natural routes of transmission are likely to involve an infectious particle that is produced and shed from some tissue. Thus far, the only infectious particle identified is the GTFP-associated herpesvirus.

Clearly, the only way to resolve the question of whether the GTFP-associated herpesvirus causes GTFP is to transmit the disease with purified virus. Preliminary attempts to isolate the herpesvirus on turtle fibroblast cultures have been unsuccessful (Chapter 5). The availability of filtered GTFP extracts that have been proven by experiment to be infectious will make it possible to continue isolation

attempts, although extensive effort may be necessary to find the cell lines and culture conditions that are permissive for this virus.

Direct purification of infectious particles from tumor homogenates was attempted as a way to obtain viruses that are difficult to culture. This approach has been effective in isolating unenveloped viruses, such as papillomaviruses, that remain infectious after extraction. This approach has also been useful in isolating enveloped viruses, such as herpesviruses and retroviruses, but their infectivity is usually destroyed in the process, making it impossible to fulfill Koch's postulates. Preliminary attempts to isolate the GTFP agent directly from infectious tumor preparations, using methods developed for isolating intact papillomaviruses yielded inconclusive results (Chapter 5). No papillomavirus-like particles were detected, but they could have been present in very low concentrations or as episomal DNA. On the other hand, particles that resembled herpesvirus were detected, although the identity of these particles remains to be proven. The gradient purified fraction was not tumorigenic in a naive green turtle. Immunoelectron microscopy and nucleic acid sequence analysis of this particle-rich fraction may provide clarification.

The transmission positive tumor homogenates and purified virus-like particles obtained from them are potential sources of nucleic acid sequences from the GTFP agent that can be

cloned and sequenced, used to make molecular probes, and used in transfection experiments to identify transforming genes (oncogenes). In addition, agent specific gene sequences can be cloned into expression vectors to produce recombinant antigens for immunodiagnostic assay development.

In lieu of fulfilling Koch's postulates with purified infectious particles, a major step toward identifying the GTFP agent can be made by screening nucleic acid sequences derived from transmission positive tumor homogenates against genomic DNA libraries from normal skin-derived and GTFP-derived cell lines. Sequences shared exclusively by infectious tumor homogenates and cultured tumor cells would be likely to belong to the GTFP agent. Koch's postulates could be fulfilled at the molecular level by inducing transformation in normal cells by transfection with these agent-specific gene sequences.

In order to perform these experiments, cell lines derived from fibropapillomas and normal skin were needed. Toward this goal efforts were made to produce tumor cell lines and to develop methods to distinguish them from normal cells (Chapter 8). Although tumor-derived and normal fibroblast lines were indistinguishable in vitro, the tumor cell lines produced tumors in the ears of immunodeficient mice whereas normal cells did not. With this valuable tool for verifying the transformed phenotype, molecular approaches

to identify the etiology and understand the pathogenesis of GTFP will be possible.

Changes in the pattern of gene expression are among the earliest cellular derangements following infection or transformation. Preliminary work toward understanding the molecular pathogenesis of fibroblast transformation in GTFP was begun by comparing the patterns of PCR amplified messenger RNA sequences, displayed by size on a sequencing gel, between paired normal skin and GTFP-derived cell lines (Chapter 8). Differential message display revealed several differences in mRNA expression between these cell lines. These differentially expressed sequences must now be cloned and sequenced. It is expected that one or more of these sequences will be identified as a virus-specific oncogene product. It is also possible that other sequences will be identified as host genes whose expression has been up- or down-regulated by infection (e.g. growth factors or their receptors). Once again, Koch's postulates could be fulfilled at the molecular level if GTFP fibroblast-specific viral gene messages can be shown to be identical to sequences found in infectious tumor homogenates.

Once the GTFP agent is unequivocally identified, all effort should be focused on developing the practical immunological and molecular diagnostic tests with which to identify sub-clinically infected green turtles, monitor populations for exposure to the agent, and study the natural

history and ecology of this infectious agent. These studies will be important for future management efforts to monitor the long term demographic effects of this disease and to curtail the spread of GTFP among individuals and among populations of this endangered species.

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BIOGRAPHICAL SKETCH

Lawrence Henry Herbst was born on September 24, 1957, in Rockaway, New York. After graduating from Chaminade High School in Mineola, New York, in 1975, he entered Cornell University and received a Bachelor of Arts degree in biology in 1978. He entered the University of Miami graduate school in 1979 and received a Master of Science degree in biology in 1983. He continued his graduate studies in zoology and ecology at the University of Minnesota between 1983 and 1985. Between 1986 and 1987 he prepared for Veterinary School while working as a research biologist in the College of Veterinary Medicine at the University of Florida. He received a Doctor of Veterinary Medicine degree from the University of Florida in 1991. Upon graduating, he remained at the University of Florida and entered the Post-Doctoral Training Program in Laboratory Animal Medicine and graduate school where he has worked to become eligible for entry into the American College of Laboratory Animal Medicine while pursuing a Doctor of Philosophy in comparative pathology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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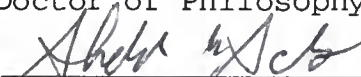
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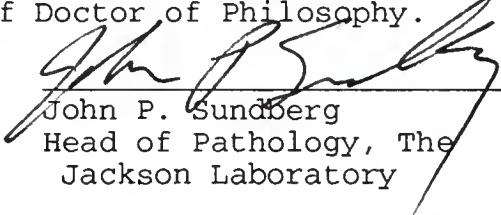
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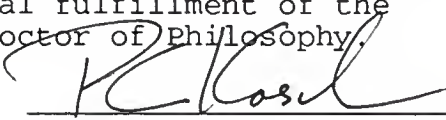
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December, 1995



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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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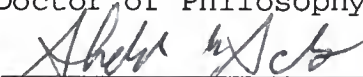
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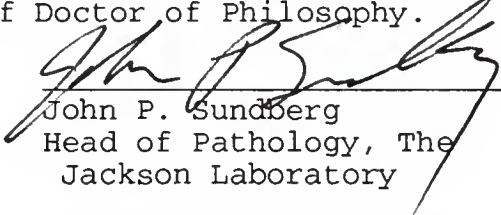
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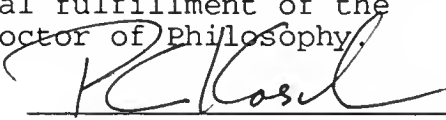
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